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(71) Applicant: PURDUE RESEARCH FOUNDATION [US/US]; Division of Sponsored Programs, Office of Technology Transfer, Room 328, 1650 Engineering Administration Building, West Lafayette, IN 47907-1650 (US).

(72) Inventors: FROST, John, W.; 1621 Dobie Circle, Okemos, MI 48864 (US). SNELL, Kristi, D.; 8 Walnut Street, Belmont, MA 02178 (US). FROST, Karen, M.; 1621 Dobie Circle, Okemos, MI 48864 (US).

(74) Agents: LAMMERT, Steven, R. et al.; Barnes & Thornburg, 1313 Merchants Bank Building, 11 South Meridian Street, Indianapolis, IN 46204 (US).

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(54) Title: DEBLOCKING THE COMMON PATHWAY OF AROMATIC AMINO ACID SYNTHESIS

(57) Abstract

Enhanced efficiency of production of aromatic compounds via the common pathway, as shown in Figure 1, of a host cell is realized by increasing the expression of enzyme species acting on substrate intermediates in identified rate-limiting reaction steps in the pathway. Prokaryotic cell transformants are described comprising exogenous DNA sequences encoding for the enzymes species, 3-dehydroquinate synthase, shikimate kinase, 5-enolpyruvoyl-shikimate-3-phosphate synthase and chorismate synthase. These transformants can be further transformed with exogenous DNA sequences encoding the enzyme species transketolase and DAHP synthase. In one embodiment of the present invention, one or more of the DNA sequences encoding the enzyme species are incorporated into the genome of the transformant.

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DEBLOCKING THE COMMON PATHWAY OF AROMATIC AMINO ACID SYNTHESIS

Field of the Invention

This invention relates to the enhancement of the efficiency of biosynthetic reactions. More particularly this invention is directed to a method for enhancing the biosynthesis of aromatic compounds in the common pathway in a host cell by genetically engineering the host cell to effectively remove the rate-limiting steps of the pathway.

Background and Summary of the Invention

The common pathway of aromatic amino acid biosynthesis, otherwise known as the shikimate pathway, produces the aromatic amino acids, phenylalanine, tyrosine, and tryptophan in bacteria and plants. The route to the aromatic amino acids consists of a common pathway that ends in the branch point molecule chorismate which is subsequently converted to phenylalanine, tyrosine and tryptophan by three separate terminal pathways. The aromatic amino acids are essential supplements to the diets of humans and animals who lack the ability to synthesize the compounds. They are also precursors for many interesting and commercially important molecules such as aspartame, a synthetic sweetener, indigo, a common dye, and L-DOPA, a drug used to combat the effects of Parkinson's disease, to name a few.

The success of any biocatalytic route to overproduce the aromatic amino acids or their derivatives from a readily available carbon source such as glucose or other sugars depends on the ability to direct a surge of carbon through the pathway of the host organism. Metabolic blocks encountered in the pathway can effect the subsequent yield and purity of products produced by the biocatalytic conversion.

Earlier approaches for increasing efficiency of production of the common pathway of aromatic biosynthesis have been described in U.S. Patent No. 5,186,056, issuing December 1, 1992, on U.S. Application Serial No. 07/652,933, filed February 8, 1991, the disclosure of which is expressly incorporated herein by reference. That patent describes a related invention directed to increasing the carbon flow into the pathway by increasing the <u>in vivo</u> catalytic activity of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase and transketolase. While the aforementioned patent teaches increasing carbon flow into the common pathway, it has been found that increased carbon flow directed into the common pathway is lost if there are one or more pathway enzymes that are not able to catalyze conversion of intermediate substrates to products at rates comparable to the rate at which those substrate intermediates are produced. Thus, there are certain rate-limiting steps in the biosynthetic pathway that work to impede the progress of the reaction steps through the pathway. The present invention removes those impediments.

The analysis of culture supernatants of the *Escherichia coli* strain D2704 (*pheA-, tyrA-, ΔtrpE-C*) using nuclear magnetic resonance spectroscopy (NMR) has identified 3-dehydroquinate synthase, shikimate kinase, 5-enolpyruvoylshikimate-3-phosphate synthase, and chorismate synthase as rate-limiting enzymes in the common pathway of aromatic amino acid biosynthesis. Transformation of *Escherichia coli* strain D2704 with exogenous DNA sequences encoding the common pathway enzyme species 3-dehydroquinate synthase (*aroB*), shikimate kinase (*aroL*), 5-enolpyruvoylshikimate-3-phosphate synthase (*aroA*), and chorismate synthase (*aroC*) resulted in a significant increase in end product production.

Brief Description of the Drawings

Fig. 1 illustrates the common pathway of aromatic amino acid biosynthesis.

Fig. 2 presents plasmid maps of pKD130A and pKD136.

Figs. 3a and 3b are bar graphs depicting the concentration of common pathway intermediates of D2704 strains of *E. coli* and the average phenylalanine and phenyllactic acid concentrations for those strains.

Fig. 4 illustrates the construction of plasmid pKD28 from plasmids pIA321 and pSU18.

Fig. 5 is similar to Fig. 4 showing construction of plasmid pKAD31.

Figs. 6a and 6b illustrate the preparation of *aro*E*aro*L plasmid pKAD34.

Figs. 7-13 are similar to Figs. 4-7 and show the construction of plasmids pKAD38, pKAD43, pKAD39, pKAD50, pKAD44, pKAD51, and pKAD42, respectively.

Fig. 14 is a graph illustrating the total accumulation of phenylalanine, phenyllactic acid and prephenic acid in culture medium of *E. coli* transformants of this invention.

Fig. 15 illustrates the construction of plasmid pAB18B from plasmids pKAD38, pKAD39, pMU377 and pGM107.

Fig. 16 illustrates the design of the synthetic cassette.

Fig. 17 illustrates the Serine biosynthetic pathway.

Fig. 18 illustrates the construction of plasmid pKAD77A.

Fig. 19 and 20 summarize common pathway end product accumulation data for several *E. coli* transformants.

Fig. 21 illustrates the location of promoters and termination sequences in the synthetic cassette.

Figs. 22-31 show the construction of plasmids pKAD69, pKAD70, pKAD68, pKAD49, pKAD62A, pKAD73, pKAD74, pKAD72A, pKAD72B, pKAD76A and pKAD80A, respectively.

Detailed Description of the Invention

In accordance with this invention there is provided a method for enhancing the production of an aromatic compound in a host cell via the common pathway of aromatic amino acid biosynthesis (the common pathway) endogenous to the host cell. In that pathway a metabolizable carbon source is converted to intermediate aromatic compounds in a multiple step reaction sequence characterized by enzyme species acting on intermediate substrates.

It has been reported that enhancing the expression of all the common pathway enzymes decreases the production of common pathway end products. However, applicants have discovered that enhancing the expression of a subset of pathway enzymes results in significant enhanced production of pathway end products. More specifically, applicants have identified the rate-limiting steps in the pathway, and have discovered that enhancing the expression of the enzyme species catalyzing the rate limiting steps (3-dehydroquinate synthase, shikimate kinase, 5-enolpyruvoylshikimate-3-phosphate synthase (EPSP synthase), and chorismate synthase) significantly enhances the production of pathway end products.

In one embodiment the biosynthesis of aromatic compounds is enhanced by transforming a host cell with recombinant DNA comprising exogenous DNA sequences encoding common pathway enzyme species, said enzyme species consisting essentially of the enzymes 3-dehydroquinate synthase, shikimate kinase, 5-enolpyruvoylshikimate-3-phosphate synthase, and chorismate synthase, and culturing the transformed cell in media containing a metabolizable

carbon source. Alternatively, enhanced expression of the enzyme species involved in the rate-limiting steps can be achieved by genetically engineering the host cell to overexpress endogenous genes for such enzyme species, either by modification of endogenous control sequences or by affecting derepression of existing expression control sequences utilizing art accepted methods.

Regardless of the exact mechanism utilized for enhancing expression of the rate-limiting enzyme species, it is contemplated that such will typically be effected or mediated by the transfer of recombinant genetic elements into the host cell. Genetic elements as herein defined include nucleic acids (generally DNA or RNA) having expressible coding sequences for products such as proteins, specifically enzymes, apoproteins or antisense RNA, which express or regulate expression of rate-limiting enzymes in the common pathway. The expressed proteins can function as enzymes, repress or derepress enzyme activity, or control expression of enzymes. Recombinant DNA encoding these expressible sequences can be either chromosomal (integrated into the host cell chromosome by, for example, homologous recombination) or extrachromosomal (for example, carried by plasmids, cosmids, and other vectors capable of effecting the targeted transformation). It is understood that the recombinant DNA utilized for transforming the host cell in accordance with this invention can include, in addition to structural genes, expression control sequences including promoters, repressors, and enhancers that act to control expression or derepression of coding sequences for proteins, apoproteins or antisense RNA. For example, such control sequences can be inserted into wild type host cells to promote overexpression of selected enzymes already encoded in the host cell genome, or alternatively, they can be used to control synthesis of extrachromosomally encoded enzymes.

The recombinant DNA can be introduced into the host cell by plasmids, cosmids, phages, yeast artificial chromosomes or other vectors that mediate transfer of genetic elements into a host cell. These vectors can include an origin of replication along with cis-acting control elements that control replication of the vector and the genetic elements carried by the vector. Selectable markers can be present on the vector to aid in the identification of host cells into which genetic elements have been introduced. Exemplary of such selectable markers are genes that confer resistance to particular antibiotics such as tetracycline, ampicillin, chloramphenicol, kanamycin, or neomycin.

A preferred means for introducing genetic elements into a host cell utilizes an extrachromosomal multi-copy plasmid vector into which genetic elements in accordance with the present invention have been inserted. Plasmid borne introduction of the genetic element into host cells involves an initial cleaving of a plasmid vector with a restriction enzyme, followed by ligation of the plasmid and genetic elements encoding the targeted enzyme species in accordance with the invention. Upon recircularization of the ligated recombinant plasmid, infection (e.g., packaging in phage lambda) or other mechanism for plasmid transfer (eg. electroporation, membrane permeabilization, microinjection, etc.) is utilized to transfer the plasmid into the host cell. Plasmids suitable for insertion of genetic elements into the host cell include but are not limited to pBR322 and its derivatives such as pAT153, pXf3, pBR325, and pBR327, pUC vectors and their derivatives, pACYC and its derivatives, pSC101 and its derivatives, and ColE1.

Suitable host cells for use in the present invention are members of those genera capable of being utilized for industrial biosynthetic production of desired

aromatic compounds. This includes any of the nonphotosynthetic eubacteria cells including prokaryotes belonging to the genera *Escherichia, Corynebacterium, Brevibacterium, Arthrobacter, Bacillus, Pseudomonas, Streptomyces, Staphylococcus, or Serratia*. More preferably prokaryotic cells are selected from the genera *Escherichia, Corynebacterium, Brevibacterium,* most preferably the genera *Escherichia*. Eukaryotic host cells can also be utilized, with yeasts of the genus *Saccharomyces* or *Schizosaccharomyces* being preferred.

More specifically, prokaryotic host cells are derived from, but not limited to, species that include *Escherichia coli, Corynebacterium glutamicum, Corynebacterium herculis, Brevibacterium divaricatum, Brevibacterium lactofermentum, Brevibacterium flavum, Bacillus brevis, Bacillus cereus, Bacillus circulans, Bacillus coagulans, Bacillus lichenformis, Bacillus megaterium, Bacillus mesentericus, Bacillus pumilis, Bacillus subtilis, Pseudomonas aeruginosa, Pseudomonas angulata, Pseudomonas fluorescens, Pseudomonas tabaci, Streptomyces aureofaciens, Streptomyces avermitilis, Streptomyces coelicolor Streptomyces griseus, Streptomyces kasugensis, Streptomyces lavendulae, Streptomyces lipmanii, Streptomyces lividans, Staphylococcus epidermis, Staphylococcus saprophyticus*, or *Serratia marcescens*. Preferred eukaryotic host cells include *Saccharomyces cerevisiae* or *Saccharomyces carlsbergensis*.

For industrial production of primary metabolites derived from chorismate (such as aromatic amino acids), deregulated mutant strains of the above recited species that lack feedback inhibition of one or more enzymes in the metabolic biosynthetic pathway are preferred. Such strains can be created by random or directed mutagenesis, or are commercially available. Examples of *E. coli* strains having DAHP synthase, prephenate dehydratase, or chorismate mutase

feedback inhibition removed are described in U.S. Patent 4,681,852 to Tribe and U.S. Patent 4,753,883 to Backman et al., the disclosures of which are incorporated herein by reference.

Shikimate kinase is one common pathway enzyme whose *in vivo* catalytic activity can be increased by mutation of a genomic locus. The *aroL* sequence encoding shikimate kinase is a part of the *aroL aroM* operon that is controlled by the *tyrR* regulon and is transcriptionally repressed in the presence of the TyrR repressor protein and either tyrosine or tryptophan. Transcriptional repression can lead to a 6.9 fold reduction in shikimate kinase activity when cells are grown in the presence of both tyrosine and tryptophan. Three *tyrR* mutants of *E. coli* strain D2704 have been constructed (KAD26B, KAD27C, and KAD29B) which do not produce the TryR repressor protein. These mutant cell lines can be used as one method of enhancing the expression of shikimate kinase. Preferably *tyrR*- strains KAD27C and KAD29B are utilized, and most preferably KAD29B is used as the *tyrR*- host cell which is further manipulated to eliminating the remaining rate limiting steps of the common pathway.

Although a *tyrR* mutation does increase levels of carbon flow through the common pathway by enhancing the expression of shikimate kinase, the effect of the *tyrR* mutation on other aspects of the cell's metabolic processes must be evaluated. In addition to the *aroL aroM* operon, eight other transcriptional units involved in either aromatic amino acid biosynthesis or transport are controlled by the *tyrR* regulon. The *aroF tyrA* transcriptional unit, encoding the tyrosine-sensitive isozyme of DAHP synthase and the bifunctional enzyme chorismate mutase-prephenate dehydrogenase, and *aroG*, encoding the phenylalanine sensitive isozyme of DAHP synthase, are transcriptionally repressed in the presence

of tyrosine and phenylalanine, respectively. Transcription of the locus encoding the third isozyme of DAHP synthase, *aroH*, has recently been shown to be under the control of the *tyrR* regulon. The transcriptional unit *mtr*, encoding an enzyme involved in tryptophan specific transport, is regulated by induction mediated by the TyrR protein whereas *tyrP*, encoding an enzyme involved in tyrosine specific transport, is regulated by both repression and induction in the presence of tyrosine and phenylalanine, respectively. In addition, the *tyrR* regulon regulates the transcription of *tyrB*, which encodes the aromatic amino transferase, *aroP*, encoding an enzyme involved in general aromatic transport, and *tyrR*, encoding the TyrR repressor protein. An additional concern arises from reports that the tyrosine operon, *aroF tyrA*, is unstable on multi-copy plasmids in *tyrR* mutants. Plasmids containing the entire *aroF tyrA* operon, when transformed into a *tyrR* mutant, are modified through insertion and deletion mutations such that the level of expression of the operon is decreased. Instability of multicopy plasmids containing *aroF* is of concern given that amplified expression of DAHP synthase, encoded by this locus, is essential to increasing the carbon flow directed into the common pathway.

In preferred embodiments of the present invention, the enhanced expression of the rate-limiting enzyme species in the host cell is achieved by transformation of the host cell with a plasmid vector comprising DNA encoding for the enzyme species 3-dehydroquinate synthase, shikimate kinase, 5-enolpyruvoylshikimate-3-phosphate synthase (EPSP synthase) and chorismate synthase. In a preferred embodiment of the present invention a prokaryotic cell is transformed with recombinant DNA to produce a prokaryotic cell transformant characterized by the expression of exogenous structural genes encoding the enzyme species transketolase (tkt), 3-

deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DAHP synthase), 3-dehydroquinate synthase (DHQ synthase), shikimate kinase, 5-enolpyruvoylshikimate-3-phosphate synthase, and chorismate synthase. Typically the exogenous structural genes are introduced into the host cell as part of one or more recombinant plasmid vectors comprising the DNA encoding for the enzyme species.

Other embodiments of the present invention include cell transformants prepared in accordance with this invention and a method utilizing such cell transformants to produce an aromatic compound biocatalytically from a carbon source. The method comprises the step of culturing a prokaryote cell transformant in media containing an assimilable source of carbon, said cell transformant comprising exogenous DNA sequences encoding common pathway enzyme species, said enzyme species consisting essentially of the enzymes 3-dehydroquinate synthase, shikimate kinase, 5-enolpyruvoylshikimate-3-phosphate synthase and chorismate synthase. The cell transformant is cultured under conditions conducive to the assimilation of the carbon source, wherein the carbon source is taken up by the cell and utilized in the common pathway. Thus applicants' invention is an improvement of their earlier work described in U.S. Patent No. 5,186,056 which discloses increasing carbon flow into the common pathway by overexpressing the enzymes transketolase and 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DAHP synthase). As disclosed in the present application, the increased carbon flow directed into the common pathway by the overexpression of transketolase and DAHP synthase is lost unless the rate-limiting steps of the pathway are removed. Applicants' invention is directed to the identification and elimination of these rate-limiting steps by enhancing the expression of common pathway enzymes consisting essentially of the enzymes 3-dehydroquinate synthase, shikimate kinase, 5-

enolpyruvoylshikimate-3-phosphate synthase and chorismate synthase.

Other embodiments of the present invention include plasmid constructs comprising structural genes encoding the rate-limiting enzymes of the common aromatic biosynthetic pathway. For example, one preferred construction comprises structural genes for 3-dehydroquinate synthase, EPSP synthase, and chorismate synthase. Most preferably the plasmid construct comprises structural genes for common pathway enzyme species, said enzyme species restricted to the rate-limiting enzymes 3-dehydroquinate synthase, shikimate kinase, 5-enolpyruvoylshikimate-3-phosphate synthase and chorismate synthase. A prokaryote cell transformed with such plasmid constructs is still another contemplated embodiment of the present invention.

As mentioned above, there have been earlier efforts to enhance the biosynthetic production of compounds derived from the common pathway in a host cell by increasing the expression of proteins catalyzing reactions in that pathway. The present invention provides for significant improvement in the efficiency of production of aromatic compounds in host cells via the common pathway. While earlier reports have taught that carbon flow can be increased into the upper end (the initial reaction sequences) of the pathway by enhancing the concentrations of transketolase alone or in combination with other enzymes in the common pathway, for example, DAHP synthase, DHQ synthase and even shikimate kinase, these references failed to teach or suggest the identification and removal of all the rate limiting steps of the common pathway. Applicants have accomplished the removal of the rate limiting steps, and thus have increased the efficiency of carbon flow through the entire pathway, by transforming the host cell with exogenous DNA sequences encoding the rate-limiting

enzyme species 3-dehydroquinate synthase (DHQ synthase), shikimate kinase, 5-enolpyruvoylshikimate-3-phosphate synthase, and chorismate synthase to increase expression of those enzymes in the host cell.

Thus the present invention can be viewed as an improvement on earlier efforts to increase the biosynthetic production of compounds derived from the common pathway, the improvement comprising the steps of (1) identifying the rate-limiting reaction steps in said pathway, and (2) increasing expression of those proteins catalyzing the identified rate-limiting steps in the pathway resulting in the removal of the rate-limiting steps. Again, the increased expression is preferably achieved in accordance with this invention by transforming the host cell to express exogenous genes encoding for said protein catalyst (enzyme) to increase concentration of the proteins in the host cell. One preferred prokaryotic cell transformant is characterized by the enhanced expression of structural genes 3-dehydroquinate synthase, shikimate kinase, 5-enolpyruvoylshikimate-3-phosphate synthase and chorismate synthase, wherein the cell comprises exogenous DNA encoding at least one of the enzyme species. The enhanced production of common pathway aromatic compounds has been shown particularly where the host cell is a strain of *E. coli* transformed to express exogenous structural genes comprising the genes for 3-dehydroquinate synthase, shikimate kinase, EPSP synthase and chorismate synthase. (See Table 1). In preferred embodiments the *E. coli* transformant further comprises exogenous DNA sequences encoding the enzyme species transketolase and DAHP synthase.

D2704, an *Escherichia coli* strain that is *pheA-, tyrA- and ΔtrpE-C* should theoretically only be able to produce chorismic acid because the terminal pathways leading to phenylalanine, tyrosine, and tryptophan are

respectively blocked (Figure 1). Using this strain it was anticipated that the deblocking of the common pathway of aromatic amino acid biosynthesis in *E. coli*, when an increased surge of carbon was committed to the pathway, could be determined by measuring the increased accumulation of chorismate. However, growth of D2704 cells in rich media followed by resuspension in minimal salts accumulation media gave little or no accumulation of chorismate but yielded significant levels of phenylalanine. The production of phenylalanine can be explained by the non-enzymatic Claisen rearrangement of chorismic acid to prephenic acid followed by dehydration to produce phenylpyruvic acid. Although the enzyme chorismate mutase accelerates the conversion of chorismate to prephenate by 2×10^6 at 37°C, the reaction can occur in the absence of the enzyme. Prephenic acid has been reported to yield phenylpyruvate non-enzymatically under mildly acidic conditions such as those produced during normal culturing of cells. With the production of phenylpyruvic acid, the microbe should be able to synthesize phenylalanine using the intact amino transferase encoded by *tyrB*. However significant amounts of phenyllactate were observed in some of the culture supernatants.

The aromatic amino transferase encoded by *tyrB* transaminates the aromatic keto acid using glutamate as the nitrogen donor and pyridoxal phosphate as a coenzyme. [Mavrides, C. In *Methods in Enzymology*; Academic: San Diego, **1987**, *142*, pp. 253-267.] The production of phenyllactic acid could be due to insufficient supplies of glutamate in the cell to completely transaminate all of the phenylpyruvic acid. Reduction of phenylpyruvic acid to phenyllactate might occur to regenerate a supply of NAD^+ within the cell. An analogous reduction of pyruvate to lactic acid catalyzed by the enzyme lactate dehydrogenase [Holbrook, J.J.; Liljas, A.; Steindel, S.S.; Rossmann, M.G.

In *The Enzymes*; Boyer, P.D., Ed.; Academic Press: New York, 1975; Vol. 11, Chap. 4] is known to occur under anaerobic conditions to regenerate a supply of NAD^+ for the continued functioning of glycolysis.

The activity of the aromatic amino transferase could also be limited by the presence of the *pheA* mutation in D2704. It has been shown that the bifunctional enzyme chorismate mutase-prephenate dehydratase encoded by *pheA* interacts with the aromatic amino transferase in the presence of phenylpyruvate to form a complex in *E. coli* [Powell, J.T.; Morrison, J.F.; *Biochem. Biophys. Acta*, **1979**, 568, 467-474]. Since D2704 is *pheA*-, it should be unable to produce the chorismate mutase-prephenate dehydratase enzyme necessary for complex formation. Although the role of the enzyme-enzyme interaction has not been determined, the possibility exists that the inability to form the complex could affect aminotransferase activity resulting in the buildup of phenylpyruvic acid within the cell. Although the above theories are plausible, the reason for phenyllactate accumulation has yet to be determined experimentally. However it is safe to assume that phenyllactate accumulation represents deblocked glucose equivalents from the common pathway. Therefore the successful removal of metabolic blocks from the common pathway of aromatic amino acid biosynthesis was measured by the combined total accumulation of phenylalanine and phenyllactic acid in the following study. Accumulation of common pathway intermediates in the culture supernatant was used to identify enzymes that were rate-limiting steps in the flow of carbon down the common pathway using the notion that the accumulated intermediate was the substrate of a rate-limiting enzyme.

Five milliliter starter cultures of each strain were grown in LB media containing the appropriate drugs for ten hours. The starter cultures were used to inoculate one

liter cultures of LB in four liter erlenmeyer flasks with isopropyl *B*-D-thiogalactopyranoside (IPTG) (0.2 mM), chloramphenicol (20 mg/L), and ampicillin (50 mg/L) added where needed. The one liter cultures were grown for 12 hours at 37°C with agitation (250 RPM). Cells were harvested (3,000 g; 5 minutes; 4°C) and washed three times with M9 salts [M9 salts contain (per liter): 6g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl, 1g NH_4Cl] (300 mls wash for each sample). Cell pellets were resuspended in one liter of M9 accumulation media in a four liter erlenmeyer flask containing glucose (10 g), $MgSO_4$ (1 mM), and thiamine (30 mg) with the addition of chloramphenicol, ampicillin and IPTG where needed. Cells were incubated for an additional 48 hours in the accumulation media at 37°C with agitation (250 RPM). Aliquots (25 ml) were removed at 24 and 48 hour intervals and centrifuged (6,000g; 5 min; 4°C). Ten milliliters of isolated supernatant was collected from each sample and the water was removed *in vacuo*. Samples were exchanged two times with D_2O and analyzed by ^1H NMR. The sodium salt of 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid was used as the internal standard to quantify intermediates and end products produced in the accumulation. All cultures were grown in triplicate so that mean values of accumulated molecules as well as their standard deviations could be obtained.

To create a surge of carbon through the common pathway of aromatic amino acid biosynthesis, a plasmid containing transketolase, *tkt*, and the tyrosine sensitive isozyme of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DAHP synthase), *aroF*, was employed. Transketolase has been shown to increase the levels of erythrose 4-phosphate available to the cell, for use in producing aromatic amino acids while DAHP synthase is the first irreversible step of the pathway. Host cell D2704 transformed with the *tkt, aroF* plasmid pKD130A (Figure 2),

a pBR325 derivative with the ampicillin resistance gene intact and a pMB1 origin of replication, accumulated the common pathway intermediates 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP), 3-dehydroshikimate (DHS), shikimate and shikimate-3-phosphate with a total phenylalanine and phenyllactate accumulation of 5.6 $\pm$ 0.7 mM (Figure 3). Figure 3 represents the data in the form of two bar graphs: Figure 3A represents the common pathway intermediates accumulated in D2704 strains after 24 hours growth in minimal media; Figure 3B illustrates the total accumulation of phenylalanine and phenyllactate after 24 and 48 hours of growth in minimal media. For both Figure 3A and 3B strains studied include: 1) D2704/pKD130A; 2) D2704/pKD136; 3) D2704/pKD136/pKD28 4) D2704/pKD136/pKAD34; 5) D2704/pKD136/pKAD31; 6) D2704/pKD136/pKAD38; 7) D2704/pKD136/pKAD43 8) D2704/pKD136/pKAD39; 9) D2704/pKD136/pKAD51; 10) D2704/pKD136/pKAD44; 11) D2704/pKD136/pKAD50.

After incubation for 48 hours, ^{1}H NMR resonances for DAHP in *E. coli* strain D2704/pKD130A are found at δ 1.79 (dd, 13, 13 Hz, 1 H), δ 2.20 (dd, 13, 5 Hz, 1 H), δ 3.46 (dd, 9, 9 Hz, 1 H) and δ 3.83 (m, 2 H). The presence of shikimate in the culture media is shown by resonances at δ 4.41 (dd, 4, 4 Hz, 1 H) and δ 6.47 (m, 1 H). A resonance for shikimate - phosphate lies at δ 6.47 (m, 1 H). Resonances for phenylalanine are found at δ 3.14 (dd, 14, 8 Hz, 1 H), δ 3.29 (dd, 14, 5 Hz, 1 H) and δ 7.30 - 7.49 (m, 5 H). Observable resonances for phenyllactic acid are found at δ 4.27 (dd, 8, 4 Hz, 1 H) and δ 7.30 - 7.49 (m, 5 H). DHS disappeared from the accumulation media between 24 and 48 hours.

The accumulation of DAHP, DHS, shikimate, and shikimate-3-phosphate in the culture supernatant of *E. coli* strain D2704/pKD130A lead to the assignment of

3-dehydroquinate synthase (DHQ synthase), shikimate dehydrogenase, shikimate kinase, and 5-enolpyruvoylshikimate-3-phosphate synthase (EPSP synthase) respectively as rate-limiting enzymes. Although DHQ synthase and shikimate dehydrogenase had been previously identified to be rate-limiting steps in the common pathway, [Draths, K.M.; Frost, J.W.; *J. Am. Chem. Soc.* **1990**, 112, 9360-9632; Draths, K.M., Ph.D. Dissertation, Stanford University, June 1991] the identification of shikimate kinase and EPSP synthase as rate-limiting steps has not been reported in the literature.

To remove the accumulation of DAHP in the culture supernatant, a *tkt, aroF, aroB* plasmid pKD136 (Figure 2) was introduced into D2704. Using pKD136, DAHP was successfully removed from the culture supernatant resulting in an increased accumulation of DHS, shikimate, and shikimate-3-phosphate but no increase in phenylalanine and phenyllactate (Figure 3). Thus even though a rate-determining step had been removed from the pathway, no increased accumulation of end product was observed.

The lack of convenient unique restriction sites for the insertion of *aroE* into pKD136 resulted in the use of a two plasmid system for the rest of the deblocking experiments. The system consisted of pKD136 and the pSU2718/pSU2719 [Martinez, E.; Bartolome, B.; de la Cruz, F. *Gene*, **1988**, 68, 159-162] derived plasmids pSU18 and pSU19, possessing chloramphenicol resistance, a lac promoter, and a p15A origin of replication, into which the remaining deblocking genes were inserted. A pSU18 based *aroE* plasmid, pKD28, [Draths, K.M., Ph.D. Dissertation, Stanford University, June 1991] was created by isolation of a 1.6 kb fragment containing a tac promoter and the *aroE* gene from pIA321 [Anton, I.A.; Coggins, J.R. *Biochem. J.*, **1988**, 249, 319-326] followed by ligation into pSU18 as

shown in Figure 4. D2704/pKD136/pKD28 while reducing the level of DHS accumulation did not completely remove the intermediate from the culture supernatant. Shikimate and shikimate-3-phosphate were still present in the culture broth. The total production of phenylalanine and phenyllactate was reduced to 2.1 ± 0.9 mM after 48 hours of growth (Figure 3, strain 3) implying that the increased carbon flow from deblocking at *aroE* did not result in the additional accumulation of end products.

To remove the rate-limiting characteristics of shikimate kinase, both *aroL* and *aroEaroL* plasmids were constructed. *aroL* is located in a transcriptional unit with *aroM*, a gene whose function is unknown [DeFeyter, R.C.; Pittard, J. *J. Bacteriol.*, **1986**, 165, 226-232]. A 2.7 kb fragment containing the transcriptional unit had previously been isolated and cloned into pBR322 to form the plasmid pMU371 [DeFeyter, R.C.; Pittard, J. *J. Bacteriol.*, **1986**, 165, 226-232]. A one kb fragment containing *aroL* was isolated from the plasmid pMU371 and inserted into the vector pSU19 creating the 3.3 kb *aroL* plasmid pKAD31 (Figure 5). The 4.9 kb *aroEaroL* plasmid pKAD34 was obtained by manipulation of the flanking restriction sites of the *aroE* gene from pKD28 followed by its isolation and ligation into the unique XbaI and BamHI sites of pKAD31 (Figure 6).

The *aroEaroL* construct D2704/pKD136/pKAD34 (Figure 3, strain 4) was able to completely remove DHS and shikimate from the culture supernatant leaving only shikimate-3-phosphate as an accumulated common pathway intermediate. The total production of phenylalanine and phenyllactate was 3.4 ± 0.2 mM, a slight increase from the end product production of D2704/pKD136/pKD28 but still significantly smaller than the phenylalanine and phenyllactate concentrations observed with both D2704/pKD130A and D2704/pKD136 (Figure 3, strains 1, and 2).

The *aroL* construct D2704/pKD136/pKAD31 was also able to completely remove DHS and shikimate from the culture broth thereby relieving the rate-limiting characteristics of both shikimate dehydrogenase and shikimate kinase with only one overproduced gene. The rate-limiting character of shikimate dehydrogenase therefore appears to be an artifact of shikimate accumulation. The importance of the removal of shikimate from the culture media on the rate-limiting characteristics of shikimate dehydrogenase suggests that shikimate may have some inhibitory effects on the enzyme. The accumulation of shikimate-3-phosphate was still observed and the total production of phenylalanine and phenyllactate was found to be 5.6 $\pm$ 0.5 mM, the level of end product production initially observed with D2704/pKD130A and D2704/pKD136 (Figure 3). Thus upon removing the metabolic blocks of DHQ synthase, shikimate dehydrogenase, and shikimate kinase, the total accumulation of pathway end products did not significantly increase leaving the deblocked glucose equivalents unaccounted for.

EPSP has been reported [Duncan, K.; Lewendon, A.; Coggins, J.R. *FEBS Lett.*, 1984, 165, 121-127] to be an inhibitor of the forward reaction of EPSP synthase suggesting a possible explanation for the observance of rate-limiting characteristics of the enzyme. To remove the shikimate-3-phosphate from the culture supernatant, both *aroA* and *aroAaroL* plasmids were constructed. The *aroA* gene exists on an operon with *serC* which encodes 3-phosphoserine aminotransferase, a serine biosynthetic pathway enzyme. The 4.7 kb fragment encoding the *serCaroA* operon has been isolated and sequenced [Duncan, K.; Coggins, J.R. *Biochem. J.*, **1986**, 234, 49-57; Duncan, K.; Lewendon, A.; Coggins, J.R. *FEBS Lett.*, **1984**, 170, 59-63]. To create the 4.7 kb *aroA* plasmid pKAD38, a 2.4 kb *aroA* fragment was isolated from the plasmid pKD501 [Duncan, K.; Coggins, J.R. *Biochem.*

J., **1986**, 234, 49-57] and ligated into the vector pSU18 directly behind the external lac promoter (Figure 7). Removal of *aroA* from the transcriptional unit of *serCaroA* necessitates its placement behind an external promoter for expression. A rho-independent transcription terminator that is located between the *serC* and *aroA* genes and is believed to naturally attenuate *aroA* expression remains intact on the 2.4 kb *aroA* fragment since a convenient restriction site for its removal was not available. Placement of the truncated *aroA* gene with the transcription terminator behind an external lac promoter should still provide some level of overexpression of EPSP synthase. The 5.7 kb *aroAaroL* plasmid, pKAD43 (Figure 8), was created by isolation of the 2.4 kb *aroA* gene with flanking PstI and blunt ended sites and ligation into a pKAD31 vector that had been manipulated to possess equivalent sites.

Evaluation of the strain D2704/pKD136/pKAD38 revealed a significant increase in total phenylalanine and phenyllactate production producing 7.9 ± 1.3 mM after 48 hours of accumulation (Figure 3, strain 6). Pathway intermediates accumulated in the supernatant were DHS, shikimate, and shikimate-3-phosphate. The strain D2704/pKD136/pKAD43 produced 9.7 ± 0.3 mM of phenylalanine and phenyllactate with the accumulation of only one common pathway intermediate, shikimate-3-phosphate (Figure 3, strain 7). The *aroA* plasmids gave the first indication of successful conversion of deblocked glucose equivalents to end products. The inability of the *aroA* gene to completely remove shikimate-3-phosphate accumulation may result from the reversibility of the reaction catalyzed by EPSP synthase.

It has been suggested that chorismate synthase is both an irreversible and possibly rate-limiting enzyme [Pittard, A.J. In *Escherichia coli and Salmonella typhimurium*; Neidhardt, F.C., Inhgraham, J.L., Low, K.B.,

Magasanik, B., Schaechter, M., Umbarger, H.E., Eds.; American Society for Microbiology: Washington, DC 1987; Vol. 1, Chapter 24]. Rate-limiting characteristics of chorismate synthase might result in the continued presence of shikimate-3-phosphate if accumulations of EPSP are subsequently converted to shikimate-3-phosphate by EPSP synthase. In an attempt to completely remove shikimate-3-phosphate from the culture supernatant, an *aroAaroCaroL* plasmid was constructed. An *aroC* plasmid was first constructed by isolation of the *aroC* fragment flanked by SalI and blunt ended sites from pGM602 [White, P.J.; Millar, G.; Coggins, J.R.; *Biochem. J.*, **1988**, 251, 313-322], a plasmid containing a 1.69 kb fragment encoding chorismate synthase. Ligation into the unique SalI and SmaI sites of pSU19 created the 4 kb plasmid pKAD39 (Figure 9). To create the 7.4 kb *aroAaroCaroL* plasmid pKAD50, the 1.69 kb *aroC* fragment was isolated from pKAD39 as a SalI/blunt ended fragment and ligated into a pKAD43 vector that had been manipulated to contain equivalent ends (Figure 10).

The strain D2704/pKD136/pKAD50 produced 12.3 ± 2.2 mM of phenylalanine and phenyllactate (Figure 3, strain 11), a significant increase in end product production over D2704/pKD136/pKAD43. While D2704/pKD136/pKAD50 still accumulated some shikimate-3-phosphate, the total amount accumulated was less than D2704/pKD136/pKAD43. The NMR of the 48 hour D2704/pKD136/pKAD50 accumulation indicates the presence of phenylalanine by resonances at δ 3.29 (dd, 14, 5 Hz, 1 H), δ 4.0 (dd, 8, 5 Hz, 1 H), and δ 7.25 - 7.49 (m, 5 H). Resonances for phenyllactic acid are found at δ 2.88 (dd, 14, 8 Hz, 1 H), δ 4.27 (dd, 8, 4 Hz, 1 H), and δ 7.25 - 7.49 (m, 5 H). A small amount of DHS is also present in the culture broth as indicated by the presence of a resonance at δ 6.4 (d, 3 Hz, 1 H). The observed increased end product production upon the addition of *aroC* to the

deblocking plasmid has lead to the assignment of chorismate synthase as a rate-limiting enzyme with the assumption that accumulation of EPSP might be converted to shikimate-3-phosphate.

To further understand the role of chorismate synthase, plasmids containing *aroC* (pKAD39; Figure 9), *aroAaroC*, and *aroCaroL* were constructed and evaluated in the strain D2704/pKD136. The 6.39 kb *aroCaroA* plasmid pKAD44 (Figure 11) was created by the isolation of an aroA fragment with flanking PstI and blunt ended sites followed by ligation into a pKAD39 vector that had been manipulated to contain equivalent blunt-ended sites. The 5 kb *aroCaroL* plasmid pKAD51 (Figure 12) was constructed by the isolation of *aroC* as a SalI blunt ended fragment which was ligated into a pKAD31 vector that had been manipulated to contain equivalent sites. As can be seen in Figure 3, pKAD39, pKAD51 and pKAD44 (strains 8, 9 and 10 respectively) did not achieve the levels of end product accumulation that the *aroAaroCaroL* plasmid pKAD50 achieved upon insertion into D2704/pKD136. Therefore the strain D2704/pKD136/pKAD50 was determined to be the optimum strain for maximal end product production.

To determine the role of transketolase in the optimal strain D2704/pKD136/pKAD50, the gene was removed from the plasmid pKD136 by digestion with BamHI followed by religation creating the plasmid pKAD42 (Figure 13). Culturing of the strain D2704/pKAD42/pKAD50 resulted in the accumulation of large amounts of acetate and lactate resulting in cell death. To alleviate this problem, the pH of the accumulation media was monitored during the 48 hour incubation and neutralized with 5N NaOH when needed. The maintenance of a neutral pH resulted in high accumulations of prephenic acid at both 24 and 48 hour time points of D2704/pKAD42/pKAD50 possibly due to the molecule's decreased ability to rearrange to phenylpyruvate at neutral

pH. Thus to compare the amount of carbon flow successfully delivered to the end of the common pathway between the strains D2704/pKAD42/pKAD50 and D2704/pKD136/pKAD50, total amounts of phenylalanine, phenyllactic acid and prephenic acid were considered.

Figure 14 illustrates the total accumulation of phenylalanine, phenyllactic acid, and prephenic acid in *E. coli* strains D2704/pKAD42/pKAD50 and D2704/pKD136/pKAD50 after 24 and 48 hours growth in minimal media. As shown in Figure 14, the amount of end products produced by the strain D2704/pKAD136/pKAD50 was significantly larger than that produced by the strain D2704/pKAD42/pKAD50. This result shows that to successfully direct an increased surge of carbon to the aromatic amino acids and their derivatives, additional copies of transketolase can be included to increase the levels of carbon available to the common pathway as well as additional copies of genes encoding DAHP synthase, DHQ synthase, shikimate kinase, EPSP synthase, and chorismate synthase.

Applicants have identified DHQ synthase, shikimate kinase, EPSP synthase, and chorismate synthase as metabolic blocks in the common pathway of aromatic amino acid biosynthesis. The previous identification of shikimate dehydrogenase as a metabolic block is thought to be an artifact of shikimate accumulation in the culture media. Plasmid pAB18B was constructed from plasmids pKAD38, pKAD39, pMU377 and pGM107 and contains each of the genes that encode the rate-limiting enzymes (see Figure 15). The construction of plasmids pKAD38 and pKAD39, providing the source of the genes *aroA* and *aroC* respectively, is described above. The construction of plasmids pMU377 and pGM107, providing the source of the genes *aroL* and *aroB* respectively, is described in references DeFeyter and Pittard, *Journal of Bacteriology*, **1986**, 165, 226-232 and Millar and Coggins, *FEBS Lett.*,

1986, 200, 11-17 respectively, the disclosures of which are expressly incorporated herein by reference.

Both the yield and purity of the aromatic amino acids and their derivatives produced by biocatalytic processes can be increased by the use of plasmid pAB18B or by the employment of a two plasmid system in *E. coli*. In the two plasmid system, plasmid pKD136 or a functional equivalent is essential to committing an increased flow of carbon to the common pathway of aromatic amino acid biosynthesis while the plasmid pKAD50 or its functional equivalent is essential to successfully direct the surge of carbon to the end of the common pathway. The increased purity of the end products observed upon introduction of the deblocking genes *aroB, aroL, aroA* and *aroC* are readily discernible in the NMRs of D2704/pKD130A and D2704/pKD136/pKAD50. A summary of the data showing applicants successful enhancement of aromatic amino acid production by the common pathway is presented in Table 1.

Table 1. Summary of Experimental Data

E. coli strain D2704 was transformed with the following combinations of plasmids and the total accumulation of phenylalanine, phenyllactic acid and prephenic acid was measured after 48 hours growth in minimal media.

PLASMIDS	OVEREXPRESSED GENES	ACCUMULATED PRODUCT
pKD130A	*tkt, AroF*	5.6 ± .7mM
pKD136	*tkt, aroF, aroB*	5.6 ± .7mM
pKD136 + pKAD34	*tkt, aroF, aroB, aroE, aroL*	3.4 ± .2mM
pKD136 + pKAD38	*tkt, aroF, aroB, aroA*	7.9 ± 1.3

pKD136 + pKAD43	*tkt, aroF, aroB, aroA, aroL*	9.7 ± .3mM
pKAD42 + pKAD50	*aroB, aroF, aroA, aroL, aroC*	8.1 ± .5mM
pKD136 + pKAD50	*tkt, aroF, aroB, aroA, aroL, aroC*	12.3 ± 2.2mM

Replacement of plasmid-based expression with genomic-based strategies for increasing *in vivo* expression of enzymes is often desirable in biocatalytic syntheses. Reducing plasmid content in a cell diminishes the metabolic burden associated with expression of genes encoded by multicopy plasmids. Metabolic burden can translate into reduced product yields and instability of the plasmid construct. Genomic insertions also provide more space to insert new plasmid-borne genes into a cell. In one embodiment, an *E. coli* transformant is constructed wherein the *E. coli* is characterized by the enhanced expression of structural genes encoding for the enzymes 3-dehydroquinate synthase, shikimate kinase, 5-enolpyruvoyl-shikimate-3-phosphate synthase and chorismate synthase. This *E. coli* transformant comprises exogenous DNA sequences encoding at least one of the enzyme species. In one embodiment the exogenous DNA sequences are integrated into the genome of the cell. This cell transformant can be further transformed with exogenous DNA sequences encoding transketolase and DAHP synthase.

Many methods exist for genomic insertions of genetic sequences into bacterial cells. Successful insertions have been performed using circular plasmids, linear DNA fragments, and transposons as carrier vehicles.

Although plasmids are maintained in bacteria as extrachromosomal, circularized DNA, recombinational events occasionally occur such that plasmid DNA is integrated into the chromosome of the host cell. Exploitation of this rare

event provides a method for simple site-specific insertion of a gene flanked by sequences homologous to the desired insertion region in the genome. Identification of cells with integrated plasmid DNA is difficult since both freely replicating and integrated plasmids express resistance to drugs encoded by plasmid markers. The use of a non-replicative plasmid allows for the exclusive selection of integrated plasmid DNA since cells will possess drug resistance only if the plasmid resides in the genome.

Plasmids whose replication can be switched on and off at will are desirable for genomic insertions. Normal cloning and preparation of the plasmid can be performed with replication in full operation whereas genomic insertions can be accomplished under conditions where the replication machinery is inactive. Plasmids possessing a temperature sensitive replicon are capable of being manipulated in this fashion. Plasmid pMAK705 contains such a temperature sensitive pSC101 replicon, a chloramphenicol acetyltransferase (*cm*) marker conferring resistance to chloramphenicol, and a convenient multiple cloning site. The plasmid is able to replicate normally when the host cell is grown at 30°C but is unable to replicate when the host cell is cultured at 44°C. Thus genetic manipulation of the plasmids are carried out at 30°C while integration of the plasmid into the genome can be selected for at 44°C.

A synthetic cassette was designed such that the *in vivo* catalytic activities of the rate-limiting enzymes EPSP synthase, chorismate synthase, and DHQ synthase is amplified with a single genomic insertion of *aroA, aroC,* and *aroB*. In this set of experiments, the host cell was an *E. coli tyrR-* mutant which, therefore, has elevated intracellular levels of shikimate kinase. The cassette was constructed from one fragment, containing an external promoter, coding sequences for *aroA*, *aroC*, and *aroB*, and a second fragment encoding an enzyme conferring resistance to

an antibiotic (Figure 16). The strong promoter, *tac*, was chosen as the external promoter to ensure sufficient transcription of *aroA* which lacks a native promoter. The gene encoding aminoglycoside 3-phosphotransferase, conferring resistance to kanamycin, was chosen as the selectable drug marker for insertion of the synthetic cassette into the genome. Insertion of the cassette into the genome at *serA*, a locus encoding 3-phosphoglycerate dehydrogenase, was planned in order to create a serine auxotroph. Figure 17 illustrates the serine biosynthetic pathway (genetic loci *ser*A, *ser*C and *ser*B encode enzymes 3-phosphoglycerate dehydrogenase, 3-phosphoserine aminotransferase, and 3-phosphoserine phosphotase, respectively). This auxotrophy can also be used to select for successful insertion of the synthetic cassette in the genome.

When analyzing the removal of the rate limiting steps of the common pathway in the *serA* auxotrophs, supplementation of serine auxotroph is not necessary since initial growth of the strain is performed in rich medium which contains serine. Subsequent resuspension of grown cells in the minimal medium used for accumulation of organic end product does not create an environment where serine is needed since the cells are simply metabolizing glucose to form phenylalanine. However, the *serA* locus must be present in the the serine auxotrophic cells if the auxotrophic cells are grown in minimal media, as is the case for large scale biocatalytic synthesis of medicinal and industrial chemicals. Inserting the *serA* locus on a plasmid and transforming a host which is *serA*- ensures maintenance of the plasmid provided the cells are grown in minimal medium (i.e. lacking amino acid supplementation). This approach is more economical for large scale biocatalysis than the antibiotics, and plasmid-encoded resistance to these antibiotics, used to maintain the

plasmids during applicants' smaller scale biocatalytic syntheses.

The synthetic cassette was inserted into the genome of KAD29B (a *tyrR*- mutant) using homologous recombination at *serA* to generate cell lines KAD1D and KAD11D. KAD1D and KAD11D were replicate plated on minimal plates with and without serine to verify that genomic insertion of the synthetic cassette disrupted the *serA* gene (Table 2). Both colonies were unable to grow without supplemented serine signifying that site specific insertion into *serA* had occurred. Control strains JC158, a *serA* auxotroph, and starting strain KAD29B were replicate plated for comparison.

Table 2. Plate Selection for Characterization of Genomic Insertion Strains

M9 plates also contained phenylalanine, tryptophan, and p-hydroxyphenylpyruvic acid to satisfy growth requirements of strains KAD29B, KAD1D, and KAD11D.

Strain	M9/glucose	M9/glucose / serine	LB/Cm	LB/Kan
JC158*serA*-	-	+	-	-
KAD29B	+	+	-	-
KAD1D	-	+	-	+
KAD11D	-	+	-	+

The *in vivo* catalytic activities of *aroB, aroL, aroA,* and *aroC* were measured to determine the level of expression of the rate-limiting enzymes in the strains KAD29B and KAD1D (Table 3).

Table 3. Ratios of Specific Activities of Rate-limiting Enzymes in Genomic Insertion Strains Relative to Control Strain D2704.

D2704 enzyme activity values (units mg-1) are as follows: DHQ synthase, 0.0023; shikimate kinase, 0.0023, EPSP synthase, 0.0099; chorismate synthase, 0.0017. One unit is defined as one μmol of product formed per min.

Strain	DHQ Synthase	Shikimate Kinase	EPSP Synthase	Chorismate Synthase
D2704	1.0	1.0	1.0	1.0
KAD29B	1.2	52	4.1	1.1
KAD1D	4.8	7.4	10	4.9
KAD1D + IPTG	3.6	6.5	11	6.5

Insertion of the synthetic cassette into *serA*, forming KAD1D, amplifies DHQ synthase expression by 3.6-fold, EPSP synthase by 11-fold, and chorismate synthase by 6.5-fold when IPTG is added to the media to induce the *tac* promoter. The high activity of shikimate kinase observed in strain KAD29B is reduced once the synthetic cassette is inserted into the genome of the strain, probably due to a metabolic burden associated with increasing the activities of the other rate-limiting enzymes.

To evaluate the effectiveness of the synthetic cassette in expressing the rate-limiting enzymes and removing the rate-limiting steps of the common pathway, applicants utilized a system that mimics genomic insertion of a plasmid through the use of a low copy plasmid. Vector pCL1920 was chosen since it contains approximately five copies per cell and has a removable *lac* promoter. Thus insertion of the cassette into pCL1920 without its promoter would provide a low copy vector in which the only

transcription initiated would be from the *tac* promoter in the cassette. The synthetic cassette was removed as a 5.5 kb *Eco*R I fragment from pKAD72A and ligated into a 4.3 kb fragment of pCL1920, in which the promoter had been previously excised, forming the 9.8 kb low copy plasmid, pKAD77A (Figure 18).

Strain KAD29B/pKD130A/pKAD77A synthesized 9.9±1.1 mM concentration of end product after 48 h of incubation. Because the pH of the culture supernatant was observed to drop to pH 5 after 24 h of culturing, cell viability was a likely problem. A second accumulation was performed where the pH was monitored during the first 24 h incubation and neutralized when needed (Figure 19). Figure 19a illustrates the accumulation of pathway intermediates DHS and S3P, whereas Figure 19b illustrates the combined accumulation of end products, phenylalanine, phenyllactic acid and prephenic acid in *E. coli* strain 1 (D2704/pKD136/pKAD50) and strain 2 (KAD29B/pKD130A/pKAD77A). Under pH controlled conditions, (pH was monitored during the first 24 hours of culturing and neutralized when needed) KAD29B/pKD130A/pKAD77A produced 12.4±1.4 mM of end products after 24 h of incubation (Figure 19b, strain 2), a value comparable to the accumulation observed with D2407/pKD136/pKAD50 (Figure 19b, strain 1). All of the glucose in the culture supernatant was metabolized during the first 24 h of minimal medium incubation and the only accumulated common pathway intermediate in the culture supernatant at 24 h was DHS (Figure 19a, strain 2).

Strains KAD1D/pKD130A and KAD11D/pKD130A have exogenous sequences encoding the enzyme species 3-dehydroquinate, EPSP synthase and chorismate synthase integrated into the genome of the cell. Additional *E. coli* strains may be constructed wherein one or more of the exogenous DNA sequences encoding the enzyme species

transketolase, DAHP synthase, 3-dehydroquinate synthase, EPSP synthase and chorismate synthase have been integrated into the genome of the cell. Strains KAD1D/pKD130A and KAD11D/pKD130A were constructed to test the ability of the genomic insertion to remove accumulations of substrates of rate-limiting enzymes and increase accumulations of end products. Figure 20a illustrates the accumulation of pathway intermediate DHS, whereas Figure 20b illustrates the combined accumulation of end products, prephenic acid, phenyllactic acid and phenylalanine in *E. coli* strain 1 (KAD29B/pKD130A/pKAD77A), strain 2 (KAD1D/pKD130A) and strain 3 (KAD11D/pKD130A). The pH of all strains was monitored during the first 24 hours of culturing in minimal media and neutralized when needed. Strain KAD1D/pKD130A accumulated 9.3$\pm$0.6 mM (Figure 20b, strain 2) while KAD11D/pKD130A accumulated 10.5$\pm$0.8 mM of end products (Figure 20b, strain 3) after 24 h of incubation in minimal medium. No significant increase in end product was observed between 24 and 48 h for either strain. The only common pathway intermediate accumulating in the culture supernatants of KAD1D/pKD130A and KAD11D/pKDS130A was DHS. Both strains metabolized all of their glucose within the first 24 h of incubation and produced slightly less end product than the 12.4$\pm$1.4 mM observed in KAD29B/pKD130A/pKAD77A (Figure 20b, strain 1).

Comparison of the *in vivo* catalytic activities of rate-limiting enzymes for strains KAD1D and KAD1D/pKD130A revealed a significant drop in activity upon the transformation of KAD1D with pKD130A (Table 4). A minimum twofold drop was observed for *aroB, aroA,* and *aroC* activities.

Table 4. Ratios of the Specific Activities of Rate-limiting Enzymes in Genomic Insertion Strains Compared to D2704.

D2704 enzyme activity values (units mg-1) are as follows: DHQ synthase, 0.023; shikimate kinase, 0.0023; EPSP synthase, 0.0099; chorismate synthase, 0.0017. One unit is defined as one µmol of product formed per min.

Strain	DHQ Synthase	Shikimate Kinase	EPSP Synthase	Chorismate Synthase
D2704	1.0	1.0	1.0	1.0
KAD1D + IPTG	3.6	6.5	11	6.5
KAD1D/pKD130A	1.7	4.1	2.4	3.1
KAD29B/pKD130A/pKAD77A	12	4.0	12	25
D2704/pKD136/pKAD50	2.0	41	8.3	4.2

Comparison of the activities of the strains KAD29B/pKD130A/pKAD77A, containing the low copy, plasmid-based synthetic cassette, with KAD1D/pKD130A, containing the genomic insertion, revealed that KAD29B/pKD130A/pKAD77A has higher *aroB, aroA* and *aroC* activities, possibly explaining the higher accumulation values observed when culturing this strain.

Example 1

Construction of *tyrR* Mutants of D2704

Introduction of the *tyrR*- allele into D2704 relied on phage P1 transduction. A P1 lysate was prepared from strain JB5 which is a derivative of a strain, JP2312, known to possess a mutation in the *tyrR370* allele. D2704 was infected with this phage lysate and colonies possessing

the *tyrR* mutation were selected for their ability to grow in the presence of the tyrosine analogue, *m*-D,L-fluorotyrosine. Because of repression of the *tyrR* regulon by TyrR protein in the presence of *m*-D,L-fluorotyrosine, colonies which are *tyrR*$^{+}$ can not biosynthesize tyrosine thereby preventing their growth in unsupplemented medium. Growth of colonies which are *tyrR*- is not affected by the presence of *m*-D,L-fluorotyrosine since these colonies do not produce TyrR repressor protein. Transduction of *tyrR* into D2704 is complicated by this strain's phenotype (*tyrA*-, *pheA*-, Δ*trpE-C*) which formally requires supplementation with phenylalanine, tryptophan, and tyrosine. Addition of tyrosine to the growth medium would negate the selection required for transduction of *tyrR*. This problem was circumvented by replacing tyrosine as a growth supplement with *p*-hydroxyphenylpyruvate, an intermediate directly preceding tyrosine on the tyrosine terminal pathway. Supplementation with *p*-hydroxyphenylpyruvate requires the D2704 variant to perform a transamination step before synthesizing tyrosine. The transaminase, encoded by *tyrB*, is repressed by the TyrR protein in the presence of *m*-D,L-fluorotyrosine. Therefore, colonies which are *tyrR*$^{+}$ will not be able to transaminate *p*-hydroxyphenylpyruvate in the presence of *m*-D,L-fluorotyrosine. The desired *tyrR*- colonies will be able to grow since the absence of TyrR protein will preclude repression of *tyrB* transcription.

Colonies with the ability to grow on *m*-D,L-fluorotyrosine were replicate plated on plates with and without the analog in order to verify the initial selection for strains possessing the *tyrR* mutation. Growth in the absence of tryptophan, tyrosine, and phenylalanine was also tested to verify the continued presence of *tyrA*, *pheA*, Δ*trpE-C* mutations. Three *tyrR* mutants, KAD26B, KAD27C, and KAD29B, were isolated along with a strain, KAD25A, that no longer required tyrosine supplementation to grow.

Activities of *tyrR* mutant strains were compared to control strain D2704 (Table 5) to determine the levels of derepression upon removal of regulation.

Table 5. Shikimate Kinase Activities of *tyrR*Strains Relative to D2704

Shikimate kinase activity of D2704 = 0.0023 units mg^{-1}. One unit is defined as one μmole of product formed per min.

Strain	Shikimate Kinase
D2704 (*tyrR+*)	1
KAD27C (*tyrR-*)	39
KAD29B (*tyrR-*)	52

KAD27C and KAD29B were found to be 39-fold and 52-fold derepressed respectively when compared to D2704. KAD29B was chosen as the strain for subsequent use since it possessed the highest *in vivo* shikimate kinase activity of the *tyrR-* isolates.

Example 2

Construction of plasmids encoding *aroA*, *aroC*, and *aroB*

Sequences of *aroA*, *aroC*, and *aroB* were analyzed by the computer program PC Gene to find restriction sites that could be used to flank the individual genes. Flanking restriction enzyme cut sites were chosen to avoid restriction enzyme digestion of the structural genes encoding *aroA, aroC,* and *aroB*. *Eco*R I was chosen as the site to flank the entire cassette since a naturally occurring *Eco*R I lies within *serA*. The *tac* promoter was positioned first in the cassette, directly in front of the *aroA* fragment, since it lacks a native promoter. The

sequence encoding *aroC* was positioned second followed by *aroB* and the kanamycin resistance marker.

The polymerase chain reaction (PCR) was used to assemble the synthetic cassette. Primers were designed to have a 19-20 base annealing sequence with the gene to be amplified. Additional bases were attached to the 5′ end of each primer to provide convenient restriction sites for construction of the synthetic cassette (Table 6).

Primers were used to amplify the shortest possible regions that still contained promoters, ribosome binding sites, and naturally occurring transcription termination sequences at the ends of genes. Locations of native promoters and transcriptional stops included in the cassette are shown in Figure 21.

Table 6. PCT Primers.

Gene	Primer #	Sequence
*aro*A (start)	914 SEQ ID NO:1	*Xba*I 5′GCTCTAGAGCTATTTCTGTTGTAGAGAGTT3′
*aro*A (tail)	915 SEQ ID NO:2	*Kpn*I 5′GTGGTACCCCATTTATTGCCCGTTGTTCAT3′
*aro*C (start)	916 SEQ ID NO:3	*Kpn*I 5′GTGGTACCCCGAACAATATCCGGATGTTCC3′
*aro*C (tail)	917 SEQ ID NO:4	*Asc*I 5′GGCGCGCCCCGGCACAGGTTGGGTTAT3′
*aro*B (start)	925 SEQ ID NO:5	*Asc*I 5′GGCGCGCCACGAATCCGCTGTATGAAGA3′
*aro*B (tail)	926 SEQ ID NO.6	*Sal*I 5′CACCGTCGACACCATTAACACCCCACTAAA3′

Amplification of *aroA*

Plasmid pKD501, a pAT153 derivative, was used as the source of the *aroA* gene. Primers 914 (SEQ ID NO:1, spanning base pairs 1465 through 1484) (Table 6) and 915 (SEQ ID NO:2, spanning base pairs 2775 through 2794) were designed to amplify a 1329 base pair fragment containing the coding sequence for *aroA*. Flanking regions of DNA were added to the primers to incorporate *Xba* I and *Kpn* I restriction sites at the start and tail of the gene respectively. Since it is part of an operon with *serC*, transcription of *aroA* runs off of the *serC* promoter located 1250 bases upstream of *aroA*. As a result, the amplified *aroA* PCR fragment did not contain a native promoter. A rho-independent termination sequence lies directly between the end of the *serC* coding sequence and the beginning of the *aroA* gene (base pairs 1440 through 1464). By using PCR, elimination of the termination sequence was possible. PCR using pKD501 as a circular template and the described primers resulted in a 1.3 kb band upon analysis by agarose gel electrophoresis. The coding sequence of *aroA* was isolated from the crude PCR reaction mixture by inserting the 1.3 kb *aroA* fragment into the *Xba* 1/*Kpn* I site of vector pSU19 forming plasmid pKAD69 (Figure 22).

Amplification of *aroC*

Searches upstream of the *aroC* coding sequence have failed to identify any exact matches of the -35 and -10 *E. coli* promoter consensus sequences. However, two putative sequences with limited homology have been identified. Since neither sequence has been verified experimentally, PCR primers were designed to include both sequences. Primers 916 (SEQ ID NO:3, spanning base pairs 335 through 355) (Table 6) and 917 (SEQ ID NO:4, spanning base pairs 1658 through 1676) were designed to amplify a 1341 base pair *aroC* fragment from plasmid pGM602, a 4.8 kb

pAT153 derivative. This *aroC* fragment included a potential inverted repeat (base pairs 1601 through 1629) characteristic of a rho-independent termination sequence. Flanking sequences of DNA were added to the primers to incorporate *Kpn* I and *Asc* I restriction sites at the start and tail of the gene, respectively. Several attempts to amplify *aroC* directly from the circular pGM602 template did not give the expected 1.3 kb band upon analysis by agarose gel electrophoresis. PCR using a linear 1.7 kb *aroC* template, isolated from pGM602 using an *Eco*R I/*Sal* I double digest, resulted in the expected 1.3 kb band.

Cloning of the *aroC* PCR fragment is complicated by the fact that *Asc* I is not a restriction site in any commercially available vectors. This problem was circumvented by exploiting the blunt ends typically possessed by PCR products. Specifically, digestion of the PCR *aroC* product with only *Kpn* I yielded a fragment with a *Kpn* I end and one blunt end. This fragment was then ligated into the *Kpn* I end/blunt end of linearized vector pBluescript KS$^+$ to generate pKAD70 (Figure 23).

Amplification of *aroB*

Primers 925 (SEQ ID NO:5, spanning base pairs 295 through 314) (Table 6) and 926 (SEQ ID NO:6, spanning base pairs 1619 through 1638) were designed to amplify a 1343 base pair *aroB* fragment from plasmid pJB14, a 6.5 kb pKK223-3 derivative. This fragment included the native promoter and an inverted repeat downstream of the *aroB* gene capable of forming a stem-loop structure characteristic of a rho-independent terminator. Flanking sequences were added to the primers to incorporate *Asc* I and *Sal* I restriction sites at the start and tail of the gene, respectively. Amplification of *aroB* using the described PCR primers resulted in the expected 1.3 kb band upon analysis by agarose gel electrophoresis.

Like *aroC*, the cloning of the *aroB* fragment is complicated by the fact that *Asc* I is not a restriction site in any commercially available vectors. To successfully clone the *aroB* PCR product, the *Asc* I site of *aroB* was ligated to the *Asc* I site of *aroC* from pKAD70 (Figure 24), forming a *Kpn* I/blunt end fragment. The *aroC aroB* fragment was cloned into the *Kpn* I/*Sma* I sites of pBluescript KS$^+$ to form pKAD68 (Figure 24).

Plasmids containing isolated PCR products were transformed into the corresponding auxotrophic mutant strains and checked for their ability to grow on glucose without aromatic supplementation. Plasmid pKAD68, which contained the *aroC aroB* insert, was transformed into both AB2849*aroC* and AB2847*aroB*. Plasmid pKAD69, which carried an *aroA* insert, was transformed into AB2829*aroA*. Plasmid pKAD70, which has the *aroC* locus, was transformed into AB2829*aroC*. All plasmids successfully complemented their corresponding auxotrophic strain enabling them to grow on M9/glucose plates lacking amino acid supplementation.

While complementation of the auxotrophic mutants by the PCR plasmid isolates suggested that functional genes had been cloned, enzyme activities were measured for each host strain D2704 transformed with the respective plasmid and compared to the enzymatic activity of the host strain to verify overexpression. All plasmids were found to significantly overexpress their enzyme activities compared to the control strain D2704. Plasmids pKAD68 and pKAD70 are cloned on the high copy number pBluescript KS$^+$, a derivative of the pUC plasmids, resulting in very high overexpression of the encoded enzymes.

Example 3
Synthesis of the Cassette

Cloning of the PCR products produced plasmids pKAD69, containing *aroA*, and pKAD68, containing an *aroC aroB* fragment. The *aroA* and the *aroB aroC* fragments were subsequently attached to a *tac* promoter sequence and a fragment encoding *kan* to assemble the full synthetic cassette (Figure 21).

To flank the *tac* promoter with the necessary *Eco*R I and *Xba* I restriction sites required for its placement in the cassette, a 0.3 kb *tac* promoter was isolated from plasmid pDR540 as an *Eco*R I *BamH* I fragment and ligated into the *Eco*R I *BamH* I sites of pSU18 to form pKAD49 (Figure 25).

Plasmid pMB2190 was used as the source of the marker gene expressing aminoglycoside 3'-phosphotransferase (*kan*), conferring resistance to kanamycin. Because plasmid pMB2190 contains *kan* on a *Pst* I fragment, a number of steps were required to flank the *kan* locus with *Sal* I and *Eco*R I sites. Plasmid pTRC99A-E was prepared by modifying the *Eco*R I site of pTRC99A by *Eco*R I digestion, treatment of the linearized fragment with mung bean nuclease, and subsequent ligation with T4 ligase. Reinsertion of an *Eco*R I site into pTRC99A-E at the opposite end of the multiple cloning site was performed by digestion of the plasmid with *Hind* III, blunt ending with mung bean nuclease, and attachment of synthetic *Eco*R I linkers forming pKAD61. The *kan* marker gene was removed from plasmid pMB2190 as a *Pst* I fragment and ligated into the unique *Pst* I site of pKAD61 forming pKAD62A (Figure 26). The *kan* fragment in pKAD62A was now flanked by the necessary *Sal* I and *Eco*R I sites for synthetic cassette construction.

A plasmid containing a *tac aroA* fragment was constructed by removing *tac* from pKAD49 as an *Eco*R I *Xba* I fragment and *aroA* from pKAD69 as an *Xba* I *Kpn* I fragment. The two genes were ligated into the *Eco*R I *Kpn* I sites of pSU18 forming the 3.9 kb plasmid pKAD73 (Figure 27).

A plasmid containing an *aroC aroB kan* fragment was constructed by removing the *aroC aroB* fragment from pKAD68 as a *Kpn* I *Sal* I fragment and *kan* from pKAD62 as a *Sal* I *Eco*R I fragment. The two fragments were ligated into the *Kpn* I *Eco*R I sites of pSU19 forming pKAD74 (Figure 28).

The entire cassette was pieced together by removing the *tac aroA* fragment from pKAD73 using an *Eco*R I *Kpn* I digest and the *aroC aroB kan* fragment from pKAD74 using a *Kpn* I *Eco*R I digest. Ligation of the fragments into the *Eco*R I site of pSU18 yielded plasmids pKAD72A and pKAD72B (Figure 29).

Example 4

Insertion of the Synthetic cassette into the Genome

The planned insertion of the synthetic cassette into the genomic copy of *serA* in strain KAD29B necessitated construction of a plasmid containing the synthetic cassette flanked by *serA* DNA. The gene for *serA* was isolated from plasmid pD2625, obtained from Genencor International, as a 1.9 kb *Eco*R V/*Dra* I fragment. Both restriction enzymes result in blunt ends which allows the fragment to be introduced into the blunt end *Sma* I site of vector p34E[27] forming plasmid pKAD63. The *serA* fragment was removed from pKAD63 as a *Sph* I fragment and ligated into the *Sph* I site of the temperature sensitive plasmid pMAK705 forming pKAD76A (Figure 30).

Cloning of the synthetic cassette into the *Eco*R I site of *serA* in pKAD76A was complicated by the fact that there are two other *Eco*R I sites in the plasmid (Figure 31). Thus a partial digest of pKAD76A with *Eco*R I was performed, creating a linear 7.4 kb fragment, into which the synthetic cassette, isolated from pKAD72A as an *Eco*R I fragment, was ligated. The resulting 12.9 kb plasmid, pKAD80A (Figure 31), contained the synthetic cassette

flanked by portions of the *serA* gene in a host vector containing a temperature sensitive replicon.

The synthetic cassette was inserted into the genome of KAD29B using homologous recombination at *serA*. Competent KAD29B cells were transformed with pKAD80A and integration of the plasmid into the genome was selected for at 44°C. Eleven cointegrates were isolated in this manner after a series of transformations. Excision of the plasmid from the genome was performed by growing cointegrates at 30°C in LB medium without drugs. Two more cycles of growth were carried out at 30°C by diluting the cultures (1:20,000) into fresh LB medium without antibiotics. Growth of the cointegrate strain at a temperature permissive to plasmid replication (30°C) creates an unstable environment for the integrated plasmid and a second recombinational event occurs allowing the excision of the plasmid from the genome. Recombination occurs such that the plasmid is either excised with its original synthetic cassette insert or with an intact *serA* sequence. Subsequent growth in liquid culture at 44°C resulted in loss of the excised plasmids from the progeny.

Colonies were finally selected for kanamycin resistance and chlorampheicol sensitivity at 44°C to identify cells that had retained the *kan* marker and excised the *cm* plasmid marker from the genome. Two such colonies were identified (KAD1D and KAD11D) and characterized further. Plasmid DNA preparations confirmed that no plasmid DNA remained in strains KAD1D and KAD11D.

To confirm that the cassette was inserted into the genome of strain KAD29B at *serA*, a Southern hybridization was performed. Genomic DNA was prepared from strains KAD29B, KAD1D, and KAD11D and digests of the DNA samples were electrophoresed on an agarose gel. DNA from the gel was transferred to a Nytran membrane and probed with a ^{32}P-labeled probe of the 1.1 kb *Pvu* II/*Kpn* I fragment

of *serA*. The resulting labeled fragments observed in the autoradigraph confirmed that site specific genomic insertion of the synthetic cassette occurred at *serA* in KAD1D. An extra band observed in the *Kpn* I/*Pvu* II/ *Asc* I triple digest of KAD11D genomic DNA corresponds to incompletely digested DNA in which the synthetic cassette was inserted into *serA*. Since all other digest of KAD11D were correct, it was assumed that the synthetic cassette was inserted into the *serA* gene of KAD11D. However, due to the extra band in the KAD11D sample, analysis of genomic insertion strains focused on strain KAD1D.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Frost, John W.
Snell, Kristi D.
Frost, Karen M.

(ii) TITLE OF INVENTION: Deblocking The Common Pathway of Aromatic Amino Acid Synthesis

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Barnes & Thornburg

(B) STREET: 11 South Meridian St.

(C) CITY: Indianapolis

(D) STATE: Indiana

(E) COUNTRY: U.S.A.

(F) ZIP: 46204

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: Unknown

(B) FILING DATE: herewith

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Lammert, Steven R.

(B) REGISTRATION NUMBER: 27653

(C) REFERENCE/DOCKET NUMBER: 3220-25621

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (317) 231-7258

(B) TELEFAX: (317) 231-7433

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCTCTAGAGC TATTTCTGTT GTAGAGAGTT 30

(3) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS:
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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(4) INFORMATION FOR SEQ ID NO:3:
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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(D) TOPOLOGY: linear
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(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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(6) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGCGCGCCAC GAATCCGCTG TATGAAGA 28

(7) INFORMATION FOR SEQ ID NO:6:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CACCGTCGAC ACCATTAACA CCCCACTAAA 30

Claims:

1. A method for producing an aromatic compound biocatalytically in a prokaryotic cell transformant via the common pathway of aromatic amino acid biosynthesis endogenous to said cell transformant, said method comprising the step of

culturing the cell transformant in media containing an assimilable carbon source under conditions conducive to the assimilation of said carbon source, said cell transformant comprising exogenous DNA sequences, integrated into the genome of the cell and encoding common pathway enzyme species, said enzyme species consisting essentially of the enzymes 3-dehydroquinate synthase, shikimate kinase, 5-enolpyruvoylshikimate-3-phosphate synthase and chorismate synthase.

2. The method of claim 1 wherein the cell transformant further comprises exogenous DNA sequences encoding the enzyme transketolase and 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase.

3. An *E. coli* transformant characterized by the expression of exogenous structural genes encoding the enzyme species 3-dehydroquinate synthase, shikimate kinase, 5-enolpyruvoyl-shikimate-3-phosphate synthase and chorismate synthase, wherein said exogenous DNA sequences are integrated into the genome of the cell.

4. The *E. coli* transformant of claim 3 further characterized by enhanced expression of the enzyme species transketolase and 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase.

5. A method for producing an aromatic compound biocatalytically from a carbon source, said method comprising the step of culturing the cell transformant of claim 4 in media containing an assimilable carbon source

under conditions conducive to the assimilation of said carbon source.

6. A method for producing an aromatic compound biocatalytically in a prokaryotic cell transformant having enhanced expression of shikimate kinase, said method comprising the step of

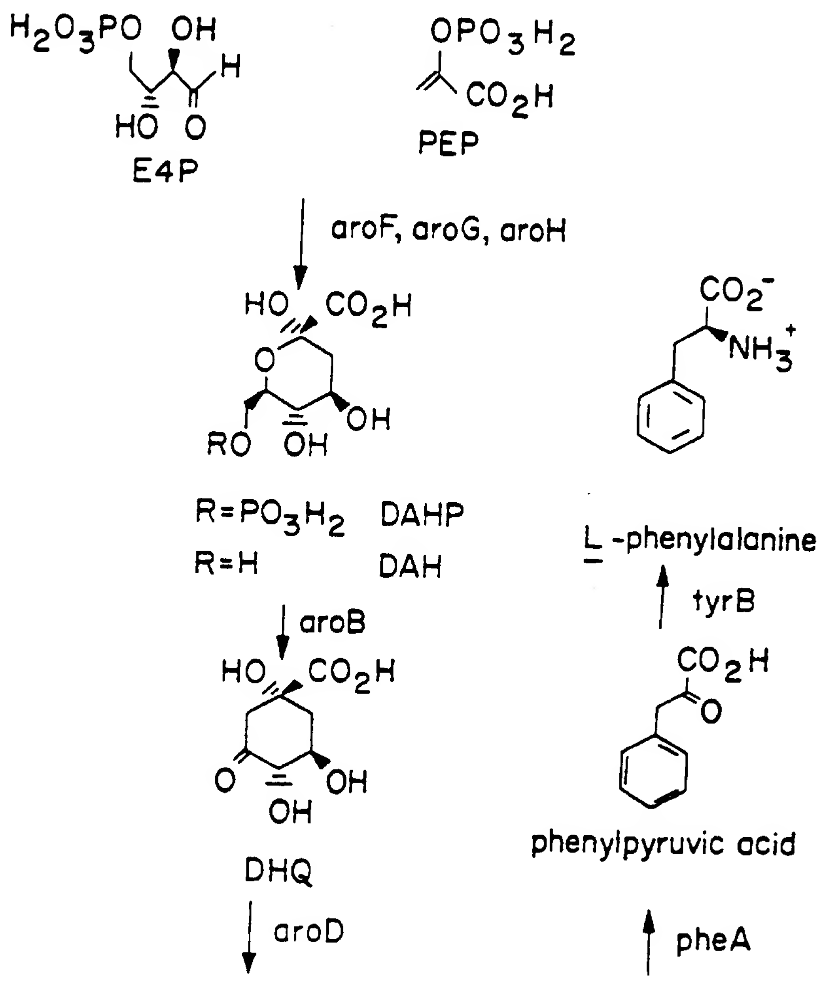
culturing the cell transformant in media containing an assimilable carbon source under conditions conducive to the assimilation of said carbon source, said cell transformant comprising exogenous DNA sequences, integrated into the genome of the cell and encoding common pathway enzyme species, said enzyme species consisting essentially of the enzymes 3-dehydroquinate synthase, 5-enolpyruvoylshikimate-3-phosphate synthase and chorismate synthase.

7. Plasmid construct pAB18B.

8. A *tyrR- E. coli* transformant characterized by the expression of exogenous structural genes encoding the enzyme species 3-dehydroquinate synthase, 5-enolpyruvoyl-shikimate-3-phosphate synthase and chorismate synthase, wherein said exogenous DNA sequences are integrated into the genome of the cell.

9. *E. coli* strain KAD29B/pKD130A/pKAD77A.

FIG. 1



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H
HO
O
E4P
OPO_3H_2
CO_2H
PEP
aroF, aroG, aroH
HO
CO_2H
O
OH
RO
OH
$R=PO_3H_2$ DAHP
R=H DAH
aroB
HO
CO_2H
O
OH
OH
DHQ
aroD
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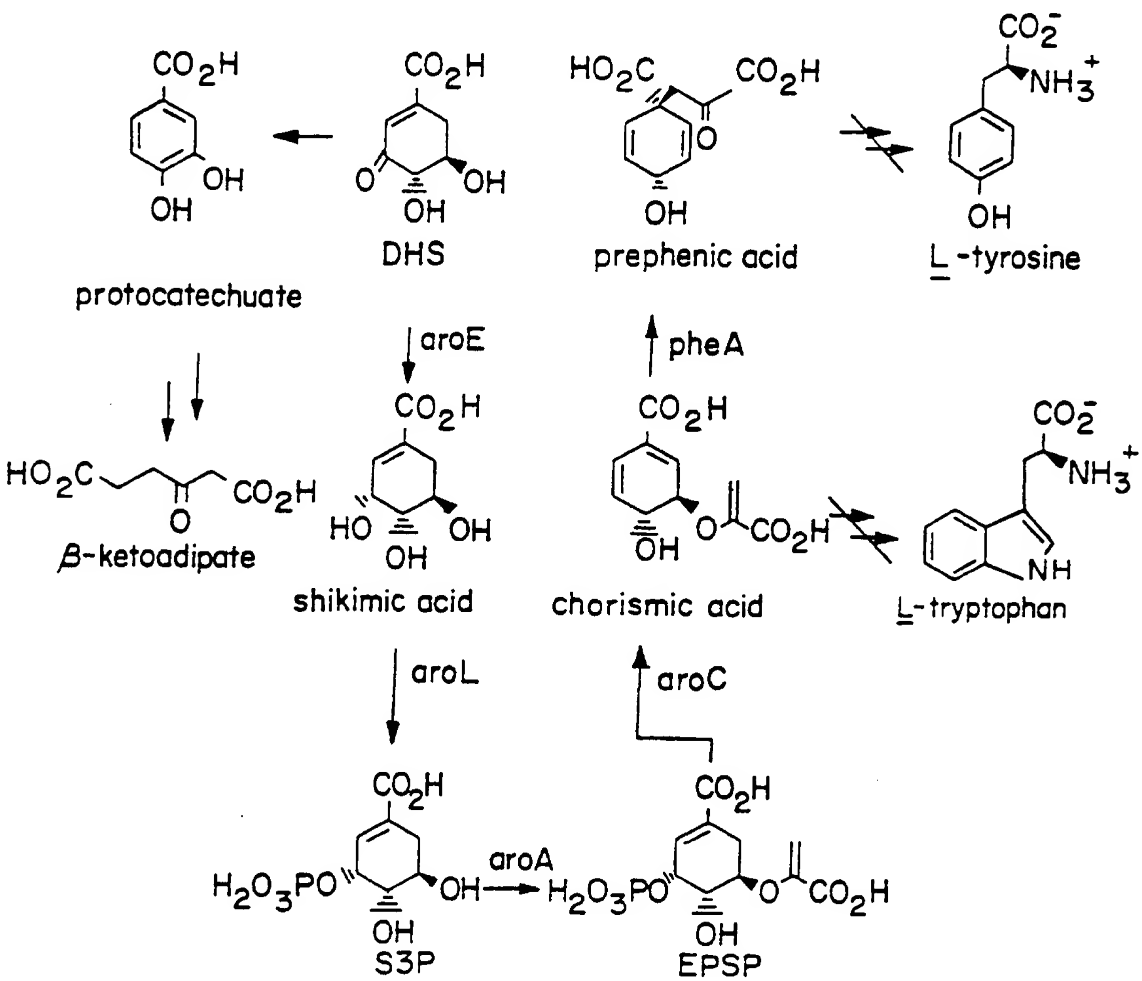
pheA

FIG.1 cont.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OH
OH
protocatechuate
CO2H
O
OH
OH
DHS
HO2C
CO2H
O
OH
prephenic acid
CO2-
NH3+
OH
L-tyrosine
aroE
pheA
HO2C
CO2H
O
β-ketoadipate
CO2H
HO
OH
OH
shikimic acid
CO2H
OH
O
CO2H
chorismic acid
CO2-
NH3+
NH
L-tryptophan
aroL
aroC
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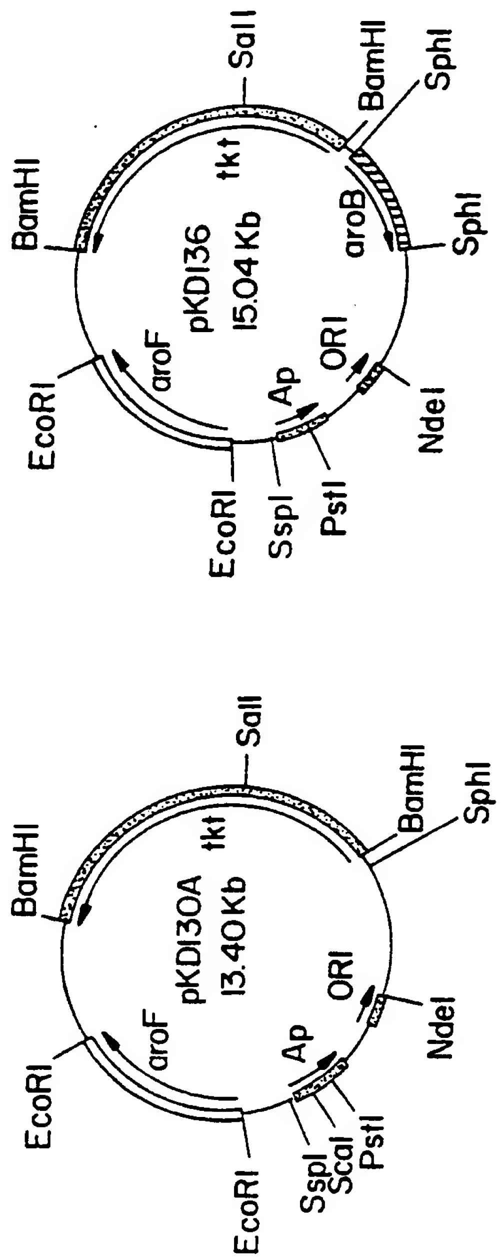
OH
OH
S3P
aroA
CO2H
H2O3PO
O
CO2H
OH
EPSP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15.04 Kb
SalI
BamHI
SphI
SphI
BamHI
tkt
aroB
ORI
NdeI
PstI
SspI
Ap
EcoRI
EcoRI
aroF
pKD130A
13.40 Kb
SalI
BamHI
SphI
BamHI
tkt
ORI
NdeI
PstI
ScaI
SspI
Ap
EcoRI
EcoRI
aroF

FIG. 2

FIG. 3A

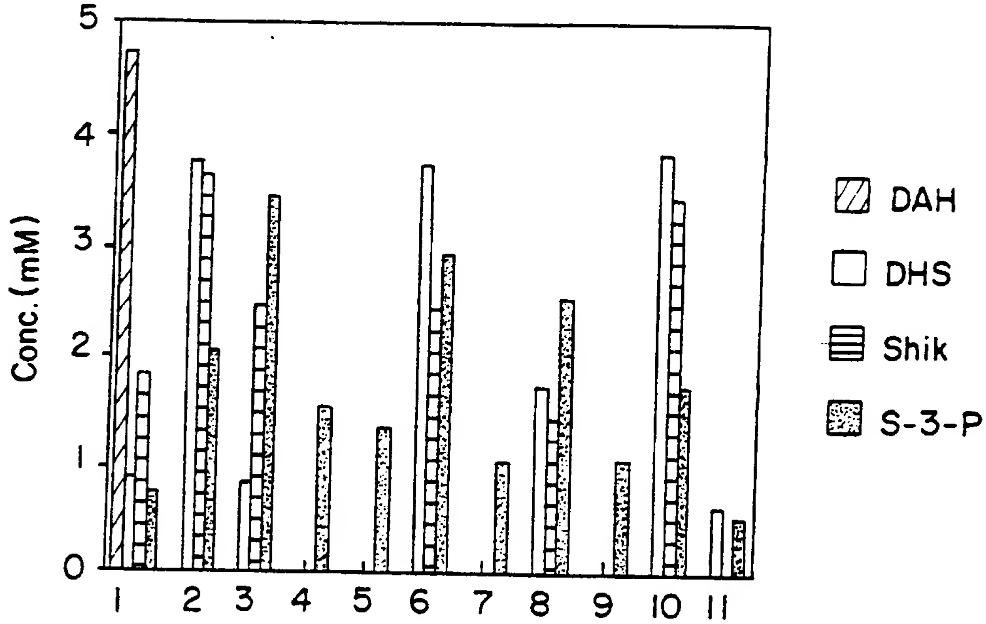

5
4
3
2
1
0
Conc. (mM)
1
2
3
4
5
6
7
8
9
10
11
DAH
DHS
Shik
S-3-P

FIG. 3B

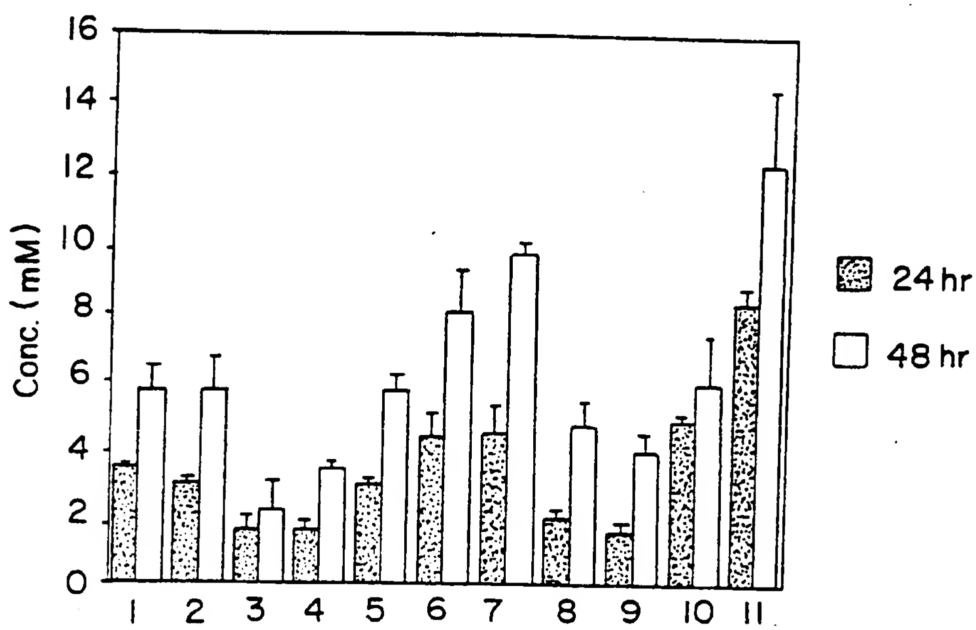

16
14
12
10
8
6
4
2
0
Conc. (mM)
1
2
3
4
5
6
7
8
9
10
11
24 hr
48 hr

FIG. 4

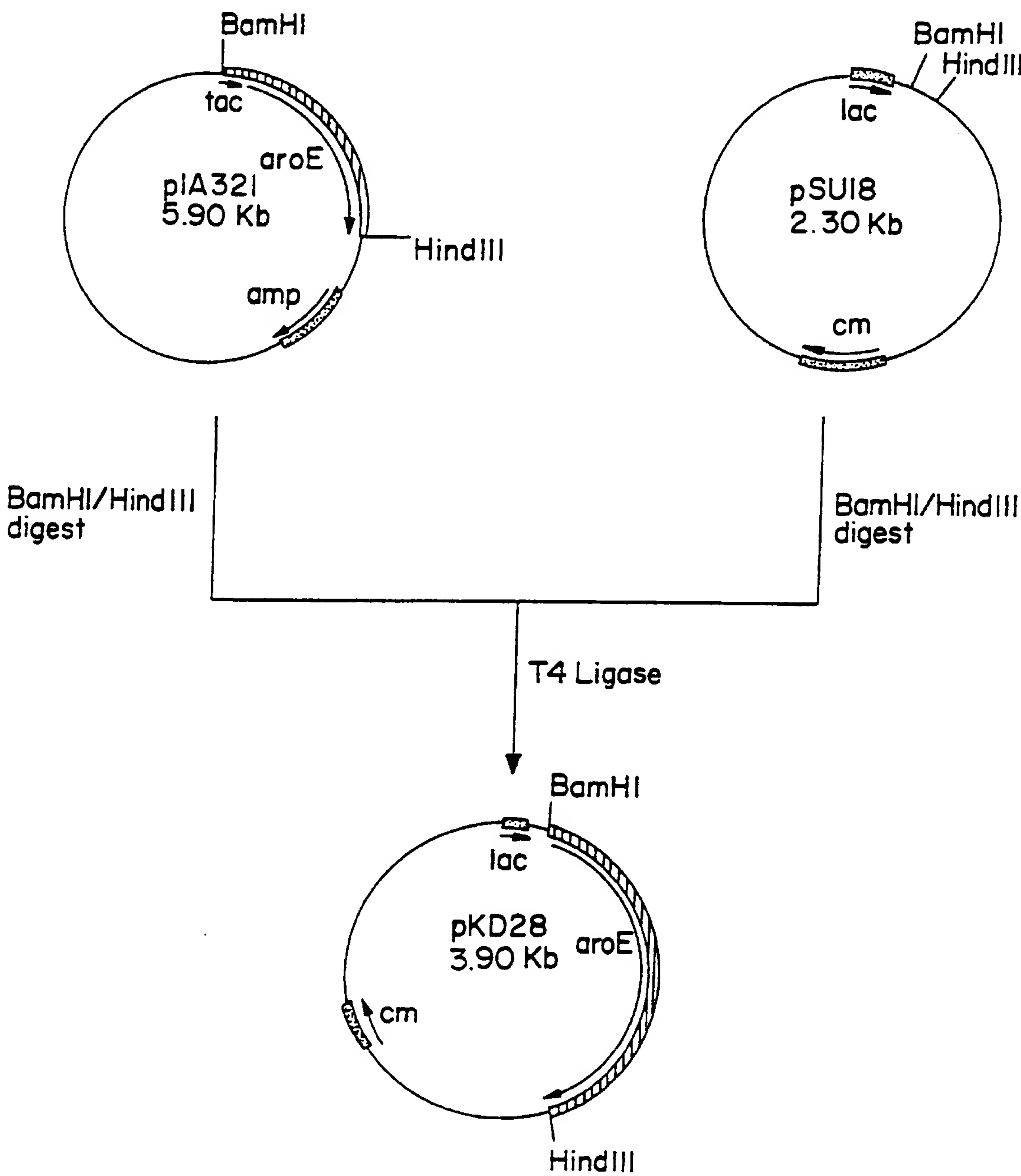
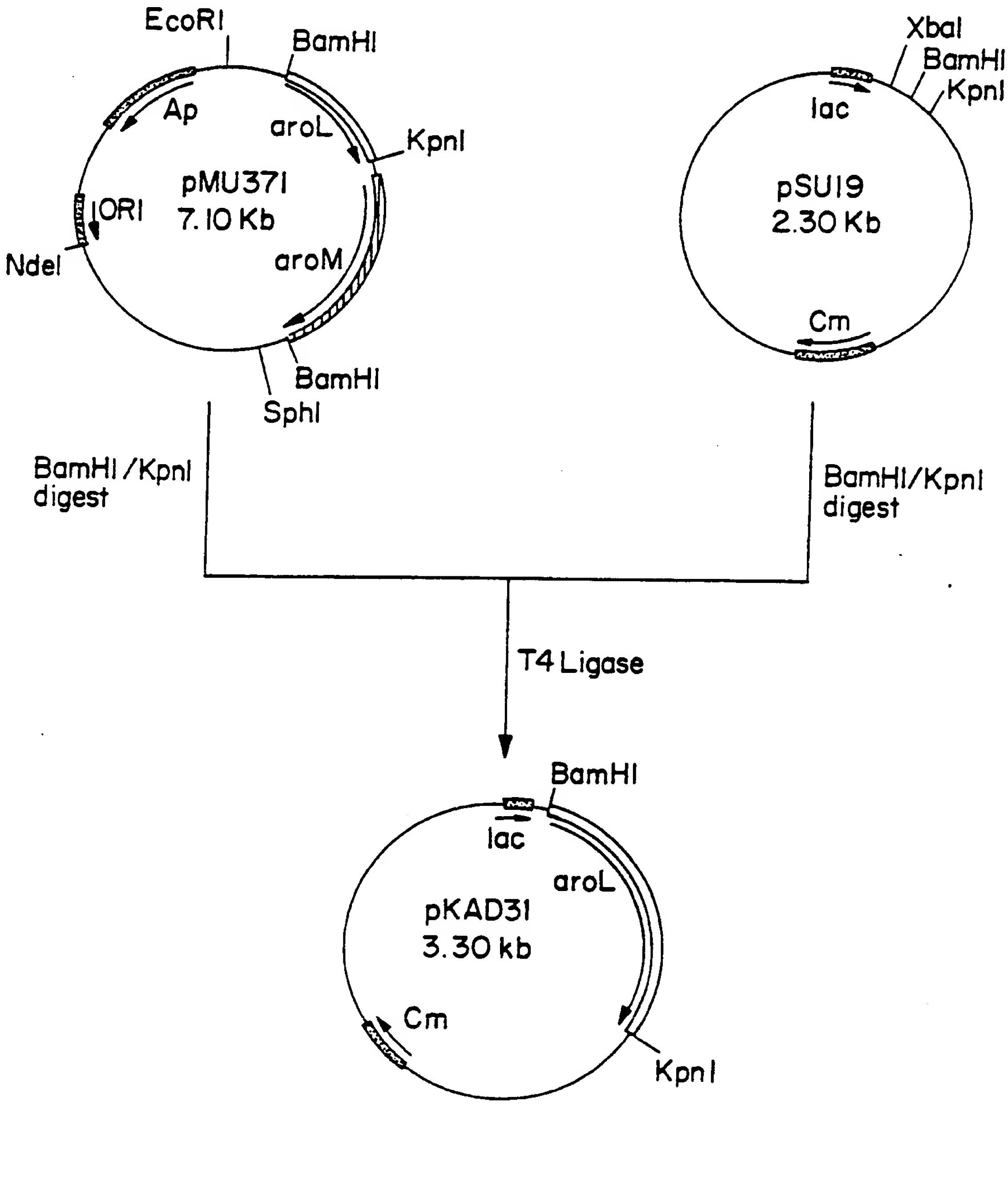

BamHI
tac
aroE
pIA321
5.90 Kb
HindIII
amp
BamHI
HindIII
lac
pSU18
2.30 Kb
cm
BamHI/HindIII
digest
BamHI/HindIII
digest
T4 Ligase
BamHI
lac
pKD28
3.90 Kb
aroE
cm
HindIII

FIG. 5


EcoRI
BamHI
Ap
aroL
KpnI
pMU371
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ORI
NdeI
aroM
BamHI
SphI
XbaI
BamHI
KpnI
lac
pSU19
2.30 Kb
Cm
BamHI/KpnI
digest
BamHI/KpnI
digest
T4 Ligase
BamHI
lac
aroL
pKAD31
3.30 kb
Cm
KpnI

FIG.6A

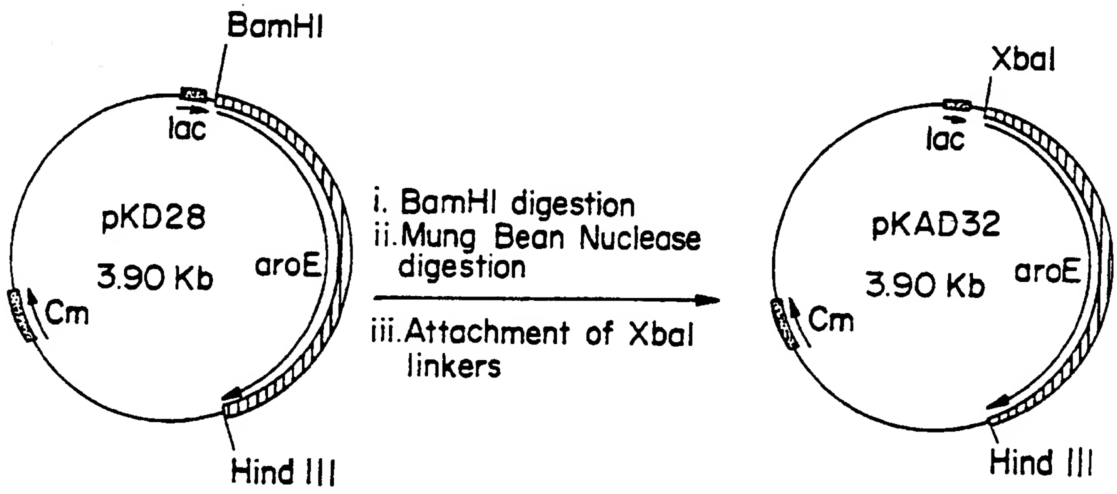

BamHI
lac
pKD28
3.90 Kb
aroE
Cm
Hind III
i. BamHI digestion
ii. Mung Bean Nuclease digestion
iii. Attachment of XbaI linkers
XbaI
lac
pKAD32
3.90 Kb
aroE
Cm
Hind III

FIG.6B

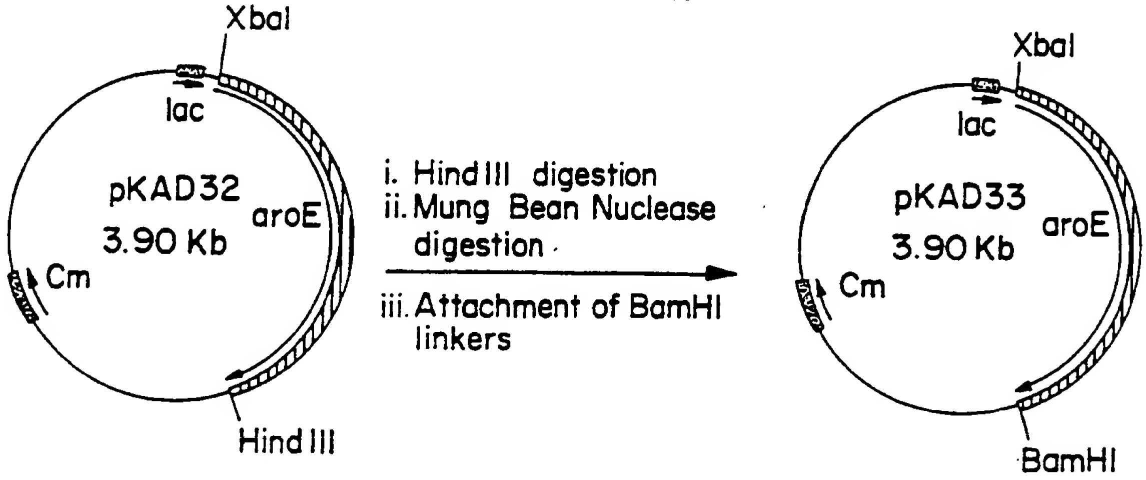

XbaI
lac
pKAD32
3.90 Kb
aroE
Cm
Hind III
i. Hind III digestion
ii. Mung Bean Nuclease digestion
iii. Attachment of BamHI linkers
XbaI
lac
pKAD33
3.90 Kb
aroE
Cm
BamHI

FIG.6C

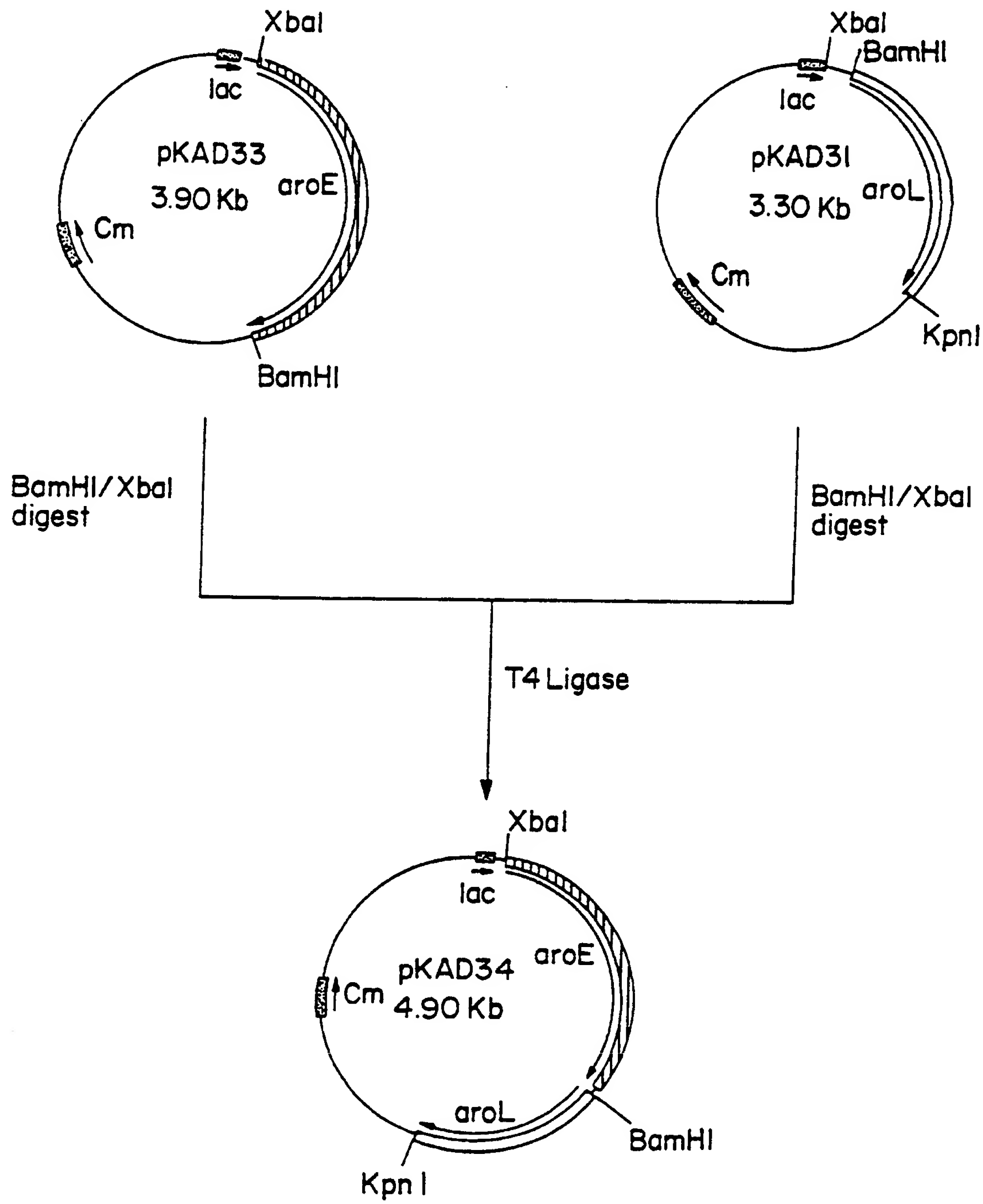

XbaI
lac
pKAD33
3.90 Kb
aroE
Cm
BamHI
XbaI
BamHI
lac
pKAD31
3.30 Kb
aroL
Cm
KpnI
BamHI/XbaI digest
BamHI/XbaI digest
T4 Ligase
XbaI
lac
pKAD34
4.90 Kb
aroE
Cm
aroL
BamHI
Kpn I

FIG. 7

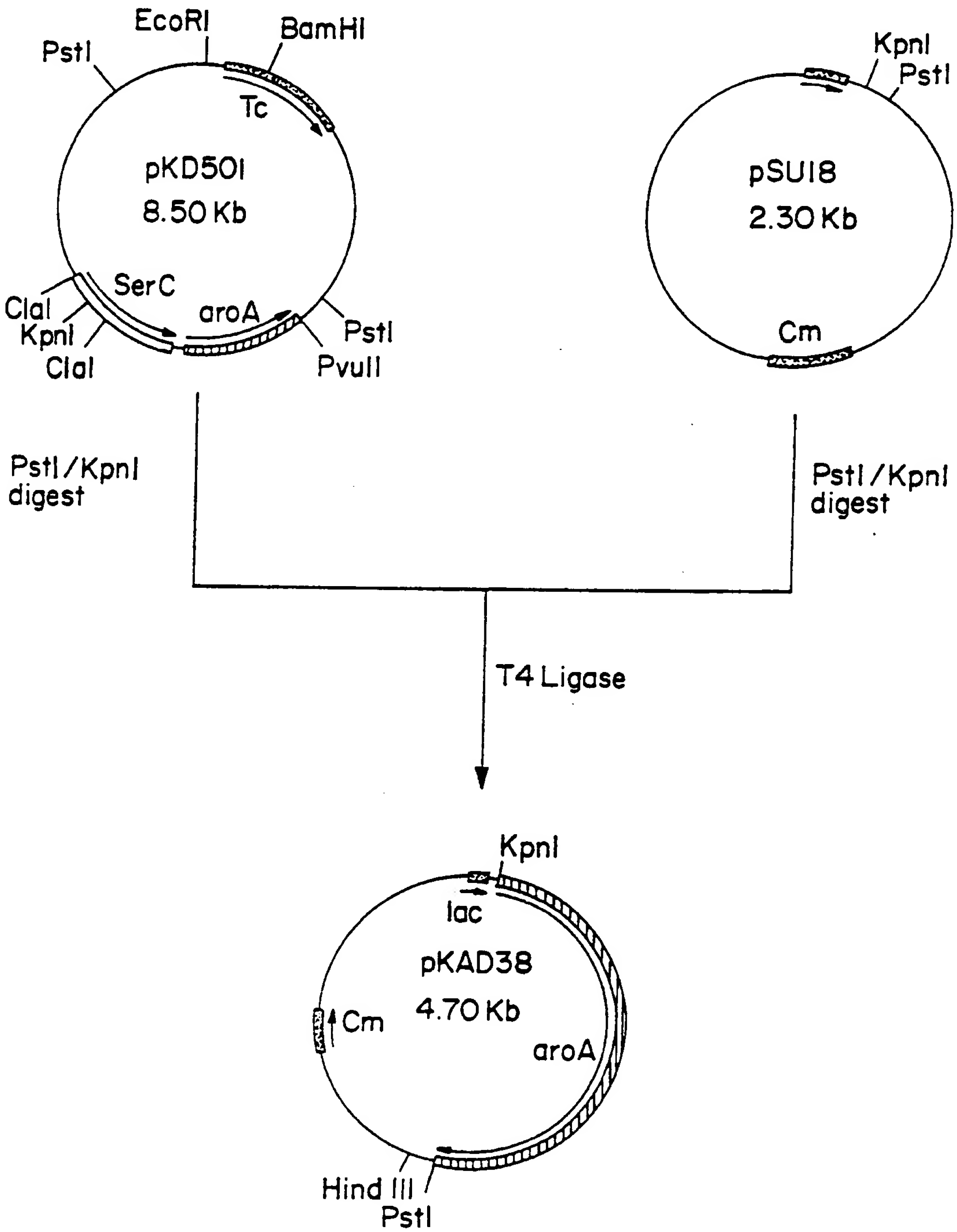

PstI
EcoRI
BamHI
Tc
pKD501
8.50 Kb
SerC
ClaI
KpnI
ClaI
aroA
PstI
PvuII
KpnI
PstI
pSU18
2.30 Kb
Cm
PstI / KpnI digest
PstI / KpnI digest
T4 Ligase
KpnI
lac
pKAD38
4.70 Kb
Cm
aroA
Hind III
PstI

FIG. 8

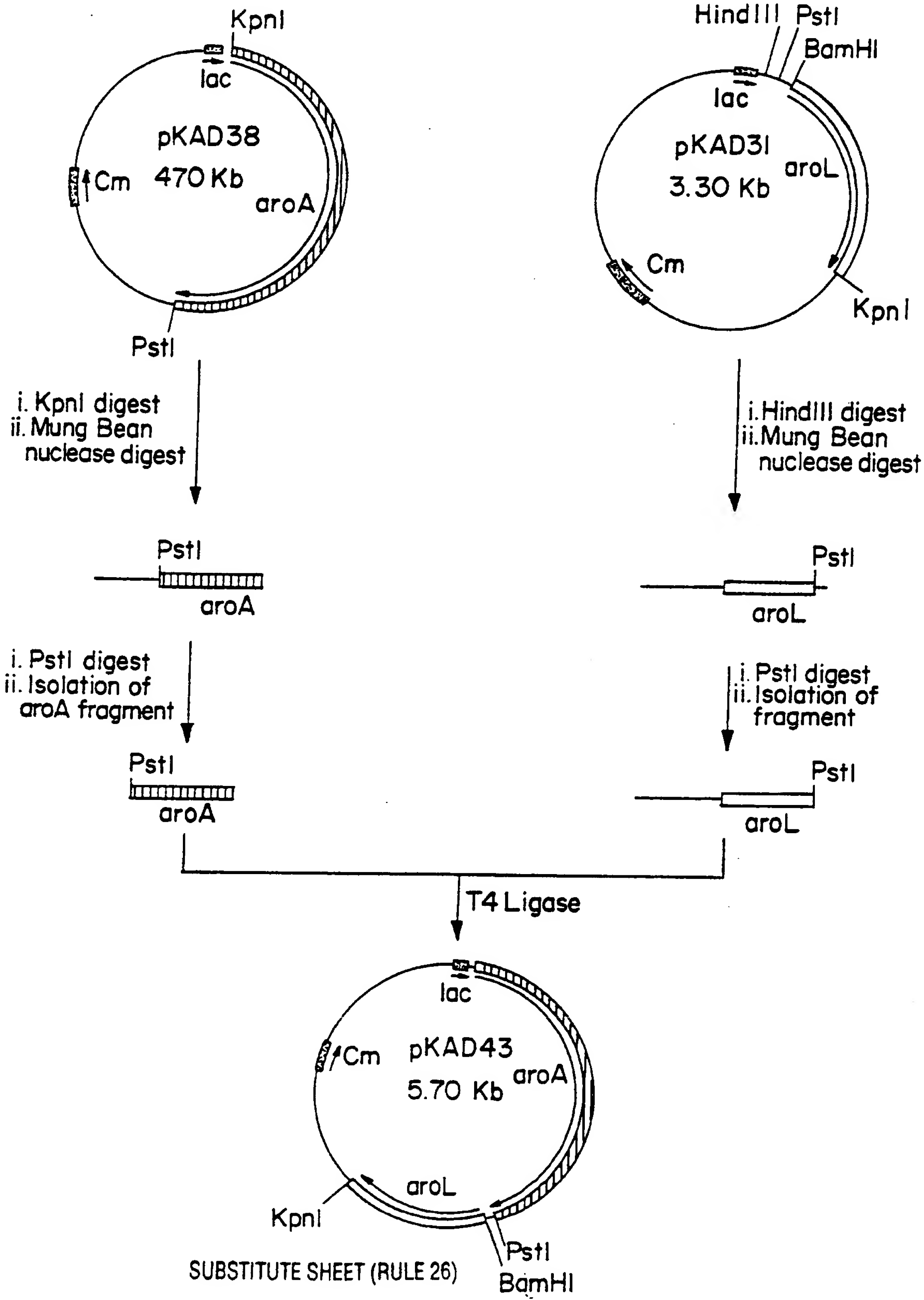

KpnI
lac
pKAD38
470 Kb
Cm
aroA
PstI
HindIII PstI
BamHI
lac
pKAD31
3.30 Kb
aroL
Cm
KpnI
i. KpnI digest
ii. Mung Bean nuclease digest
i. HindIII digest
ii. Mung Bean nuclease digest
PstI
aroA
PstI
aroL
i. PstI digest
ii. Isolation of aroA fragment
i. PstI digest
ii. Isolation of fragment
PstI
aroA
PstI
aroL
T4 Ligase
lac
pKAD43
5.70 Kb
Cm
aroA
aroL
KpnI
PstI
BamHI

FIG. 9

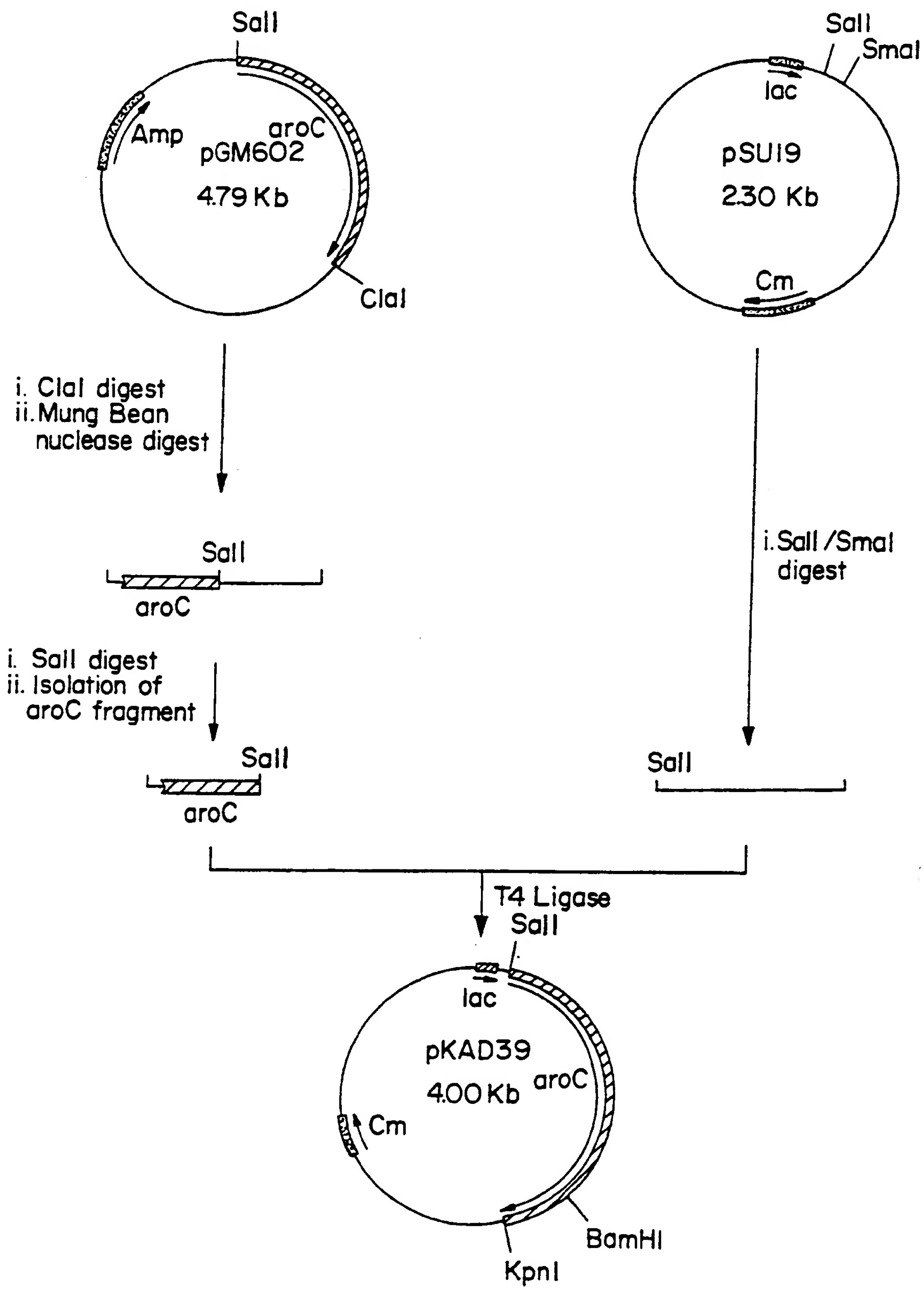

SalI
Amp
aroC
pGM602
4.79 Kb
ClaI
SalI
SmaI
lac
pSU19
2.30 Kb
Cm
i. ClaI digest
ii. Mung Bean
nuclease digest
SalI
aroC
i. SalI digest
ii. Isolation of
aroC fragment
SalI
aroC
i. SalI /SmaI
digest
SalI
T4 Ligase
SalI
lac
pKAD39
4.00 Kb
aroC
Cm
BamHI
KpnI

FIG. 10

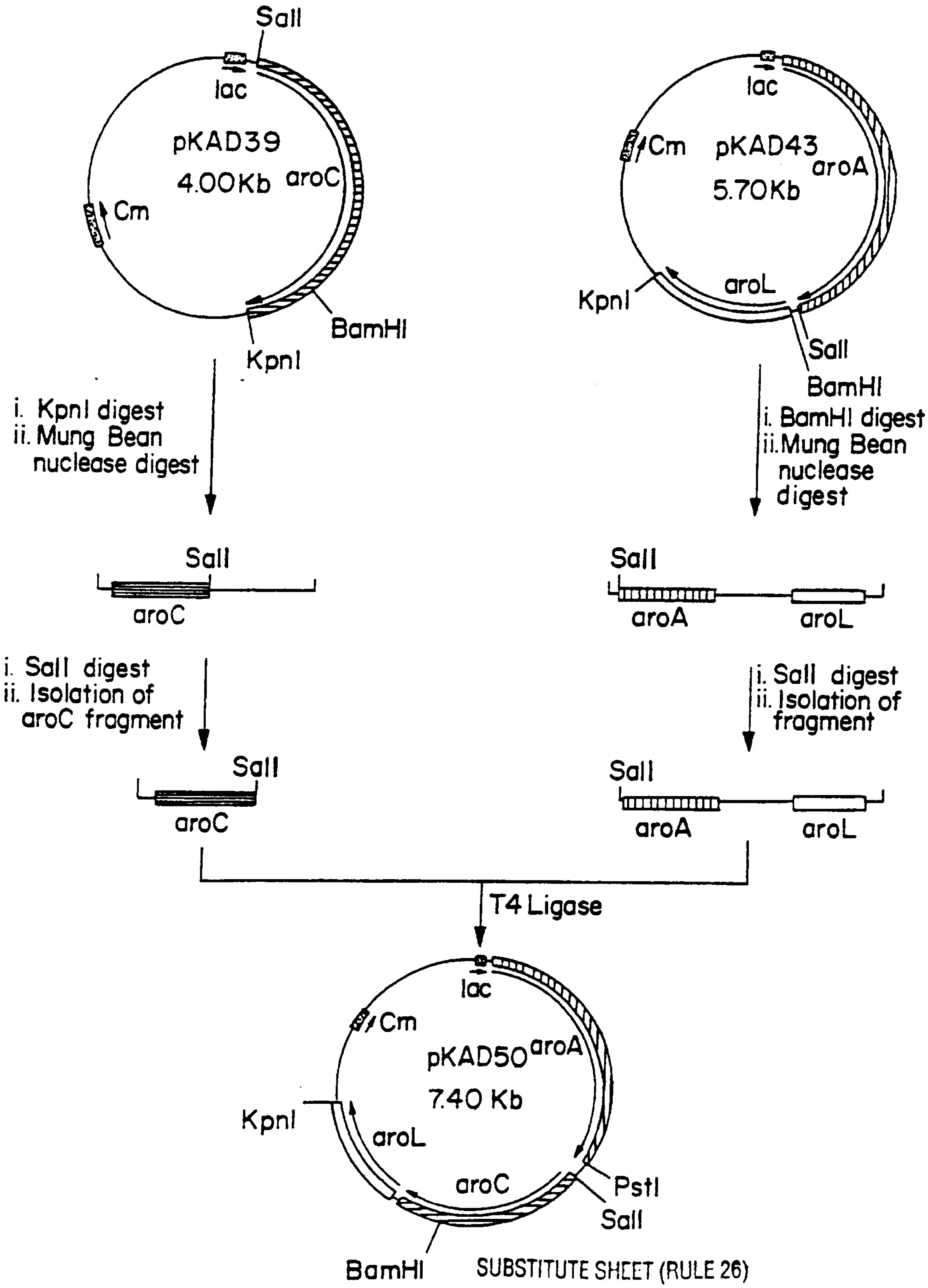

SalI
lac
pKAD39
4.00Kb
aroC
Cm
BamHI
KpnI
lac
Cm
pKAD43
5.70Kb
aroA
aroL
KpnI
SalI
BamHI
i. KpnI digest
ii. Mung Bean nuclease digest
i. BamHI digest
ii. Mung Bean nuclease digest
SalI
aroC
SalI
aroA
aroL
i. SalI digest
ii. Isolation of aroC fragment
i. SalI digest
ii. Isolation of fragment
SalI
aroC
SalI
aroA
aroL
T4 Ligase
lac
Cm
pKAD50
7.40 Kb
aroA
KpnI
aroL
aroC
PstI
SalI
BamHI

FIG. 11

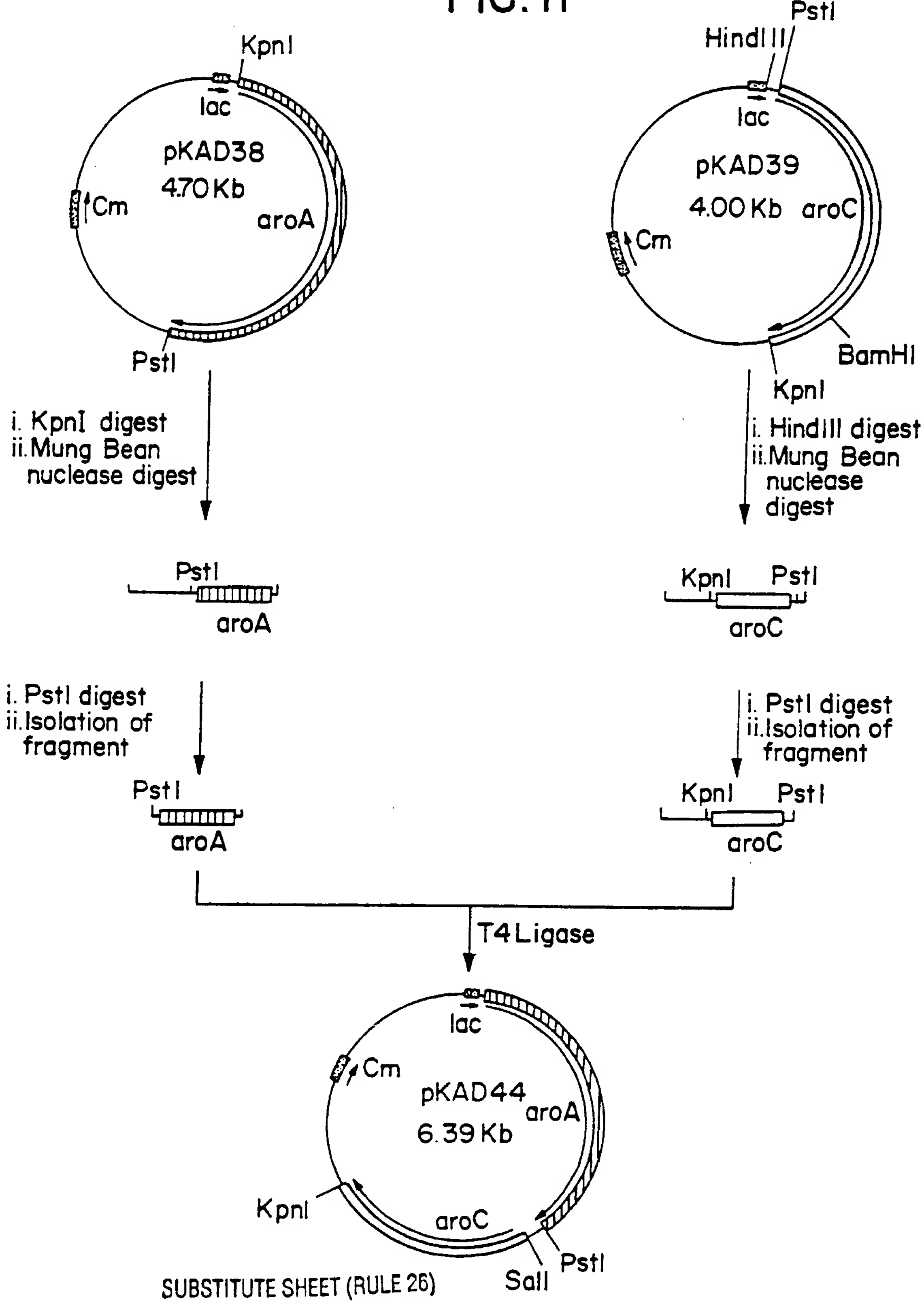

KpnI
lac
pKAD38
4.70 Kb
Cm
aroA
PstI
i. KpnI digest
ii. Mung Bean nuclease digest
PstI
aroA
i. PstI digest
ii. Isolation of fragment
PstI
aroA
PstI
HindIII
lac
pKAD39
4.00 Kb
aroC
Cm
BamHI
KpnI
i. HindIII digest
ii. Mung Bean nuclease digest
KpnI
PstI
aroC
i. PstI digest
ii. Isolation of fragment
KpnI
PstI
aroC
T4 Ligase
lac
Cm
pKAD44
6.39 Kb
aroA
KpnI
aroC
PstI
SalI

FIG. 12

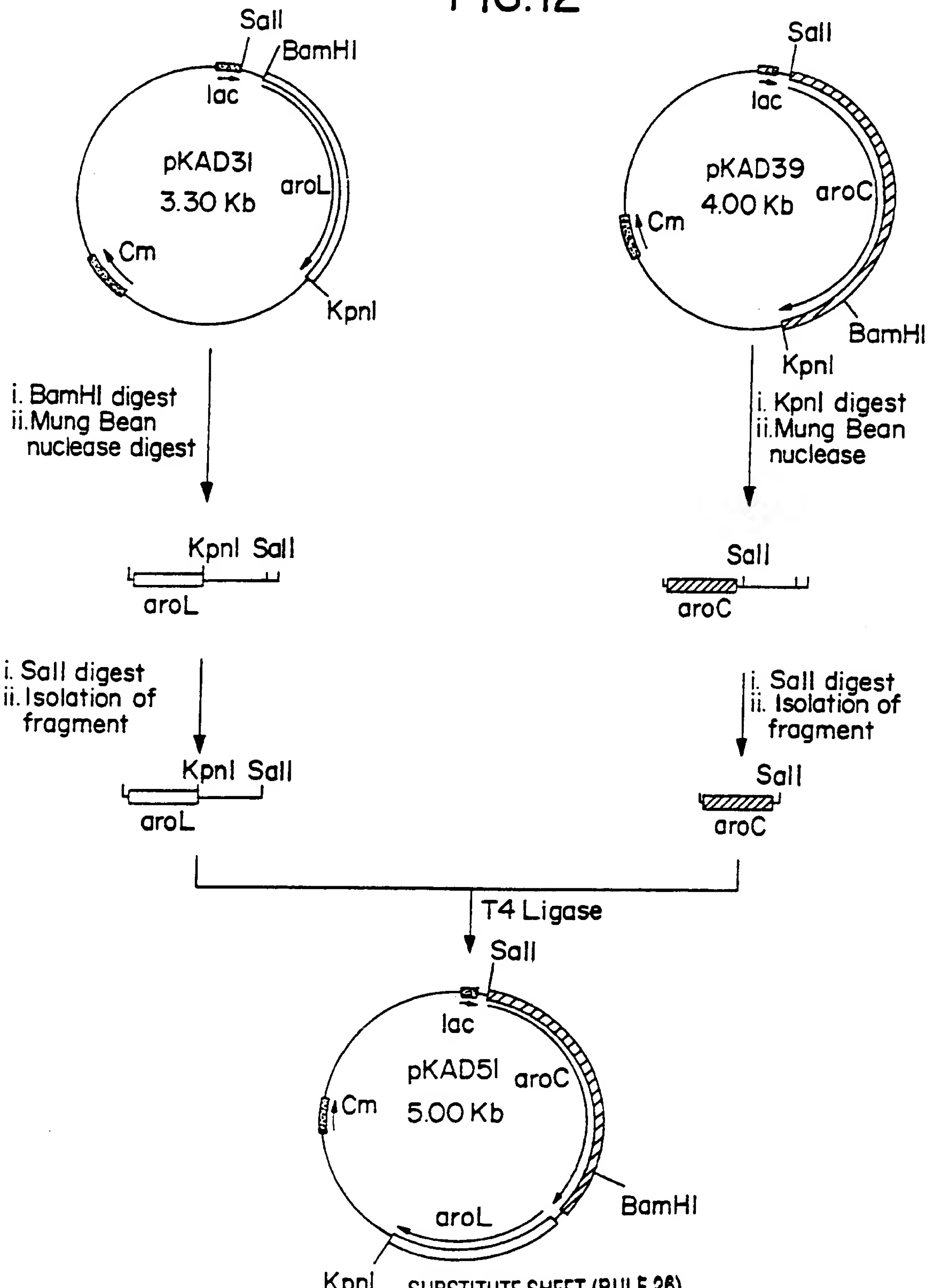

SalI
BamHI
lac
pKAD31
3.30 Kb
aroL
Cm
KpnI
SalI
lac
pKAD39
4.00 Kb
aroC
Cm
BamHI
KpnI
i. BamHI digest
ii. Mung Bean
nuclease digest
i. KpnI digest
ii. Mung Bean
nuclease
KpnI SalI
aroL
SalI
aroC
i. SalI digest
ii. Isolation of
fragment
i. SalI digest
ii. Isolation of
fragment
KpnI SalI
aroL
SalI
aroC
T4 Ligase
SalI
lac
pKAD51
5.00 Kb
aroC
Cm
BamHI
aroL
KpnI

FIG. 13

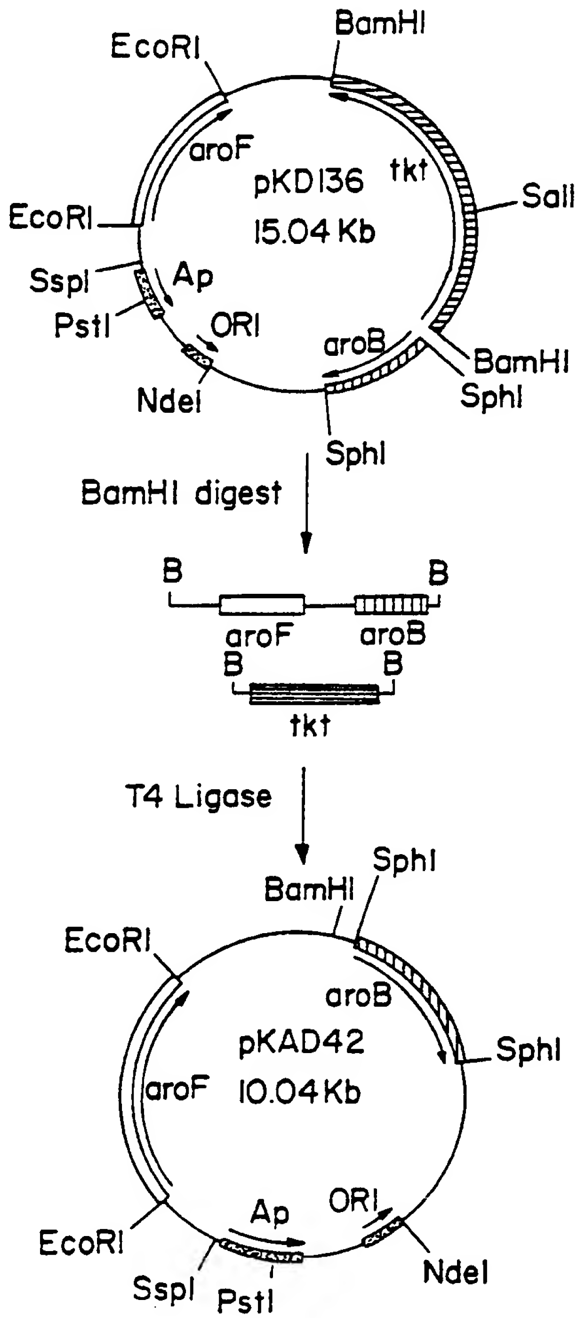
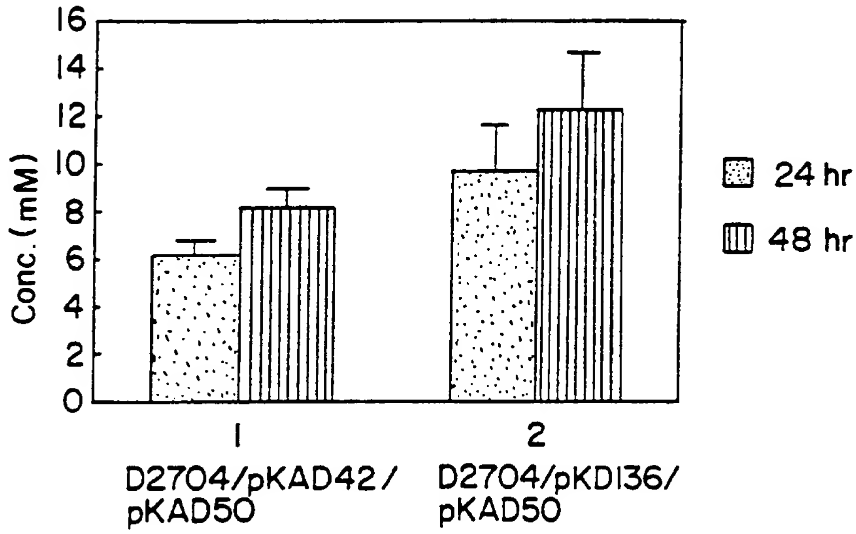

BamHI
EcoRI
aroF
tkt
pKD136
15.04 Kb
SalI
EcoRI
SspI
Ap
PstI
ORI
aroB
BamHI
SphI
NdeI
SphI
BamHI digest
B
B
aroF
aroB
B
B
tkt
T4 Ligase
SphI
BamHI
EcoRI
aroB
pKAD42
SphI
aroF
10.04 Kb
Ap
ORI
EcoRI
NdeI
SspI
PstI

FIG. 14


16
14
12
10
8
6
4
2
0
Conc. (mM)
24 hr
48 hr
1
D2704/pKAD42/
pKAD50
2
D2704/pKD136/
pKAD50

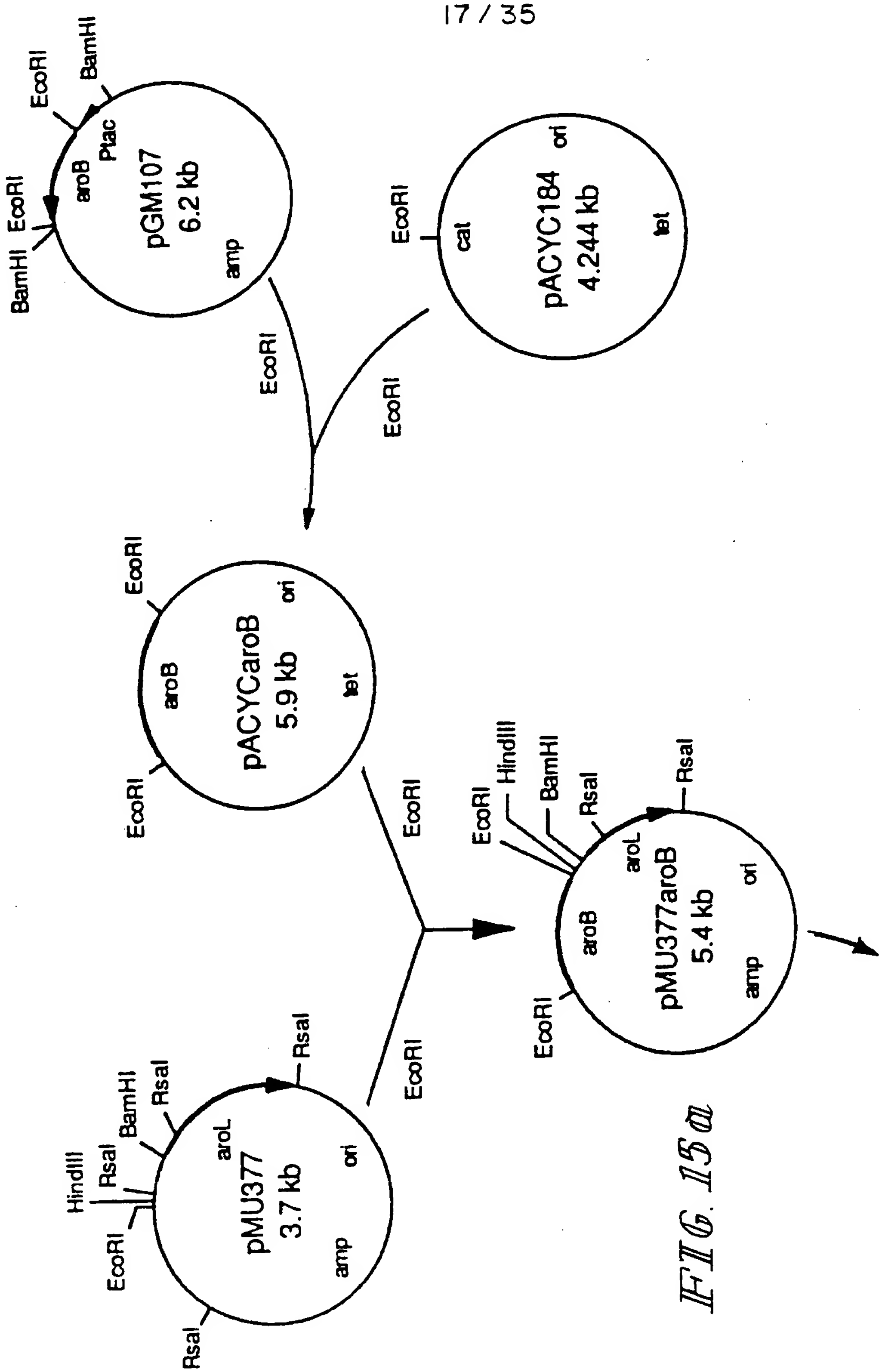

pGM107
6.2 kb
EcoRI
BamHI
Ptac
aroB
amp
pACYC184
4.244 kb
cat
tet
ori
pACYCaroB
5.9 kb
pMU377
3.7 kb
HindIII
RsaI
aroL
pMU377aroB
5.4 kb

FIG. 15a

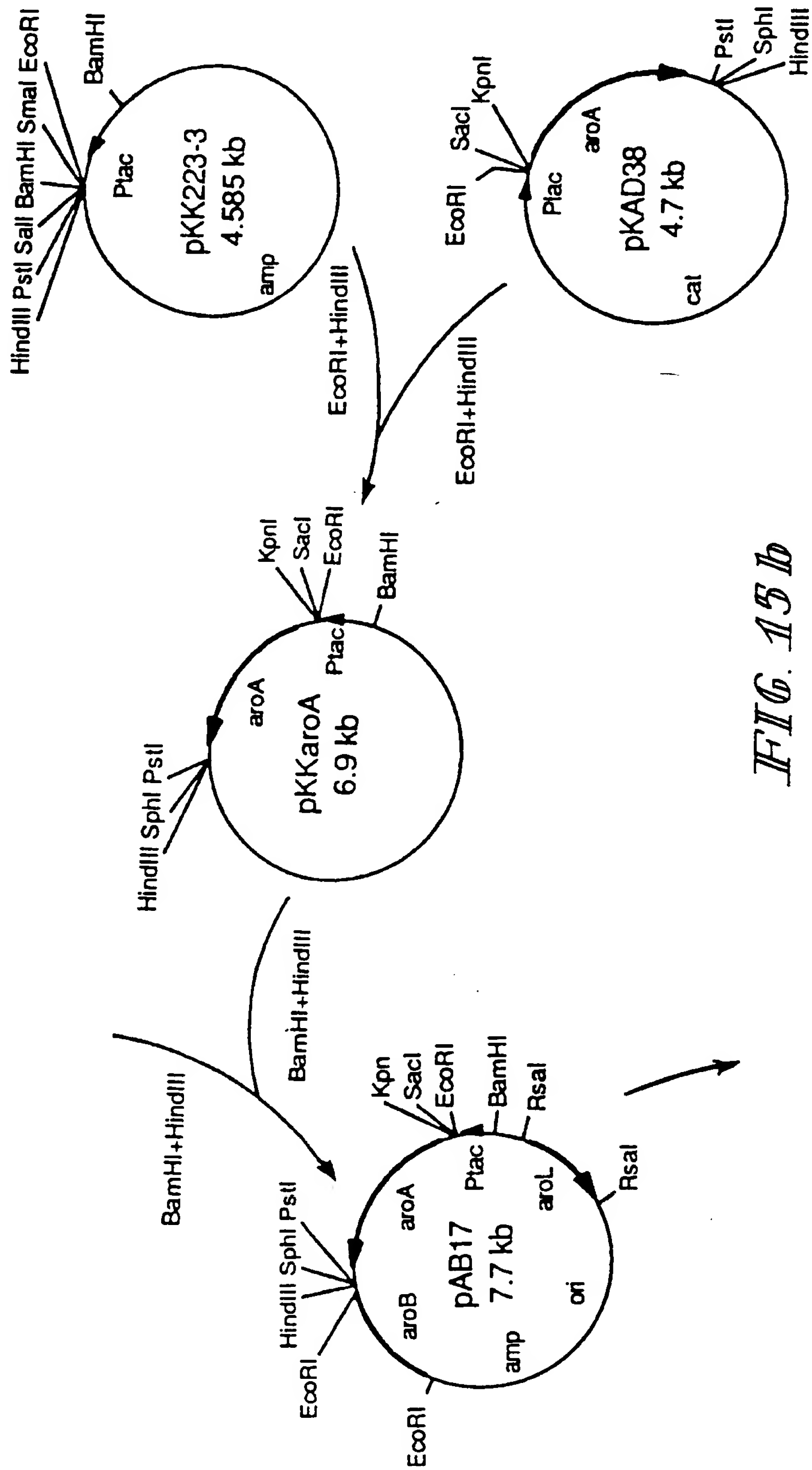
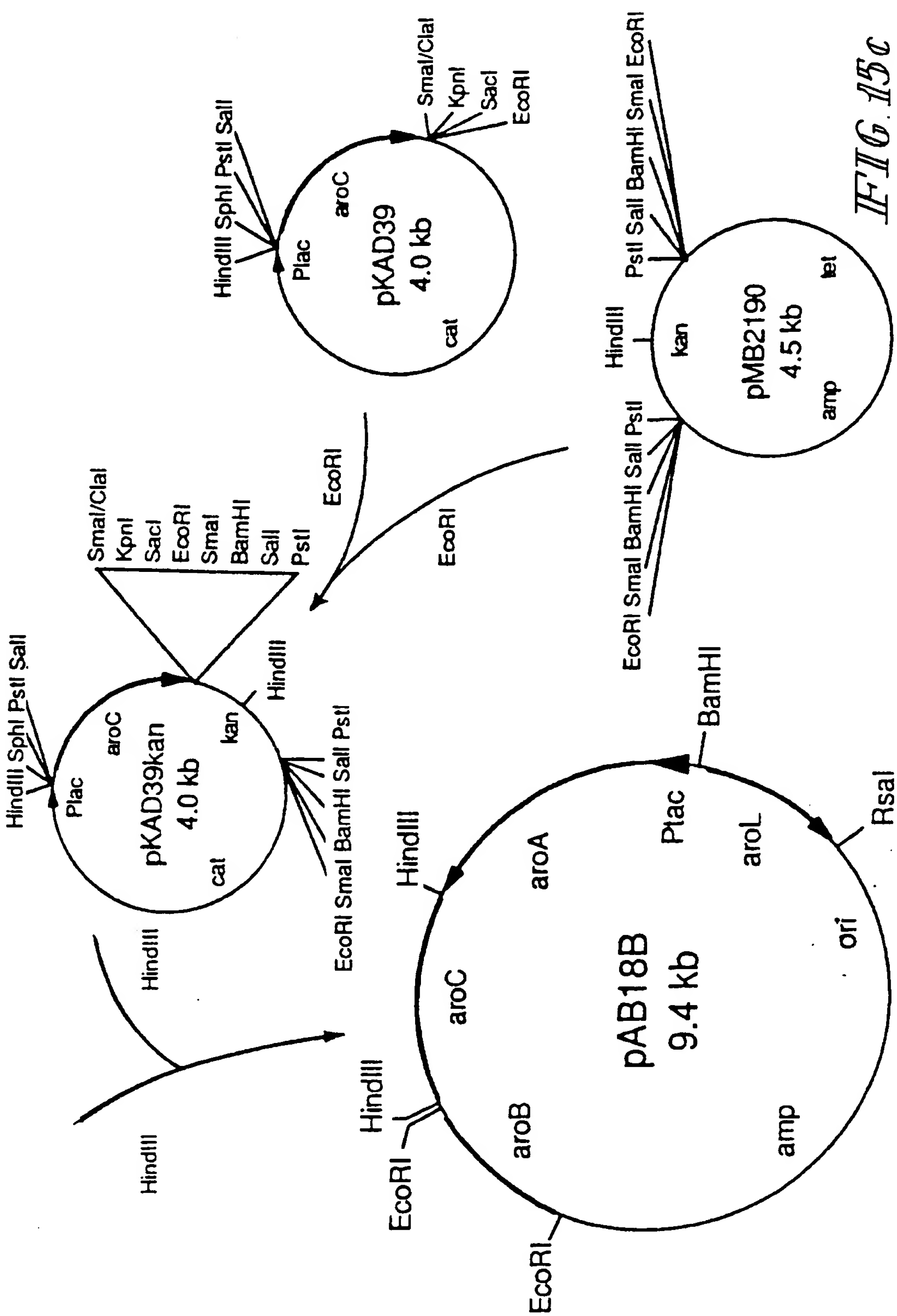
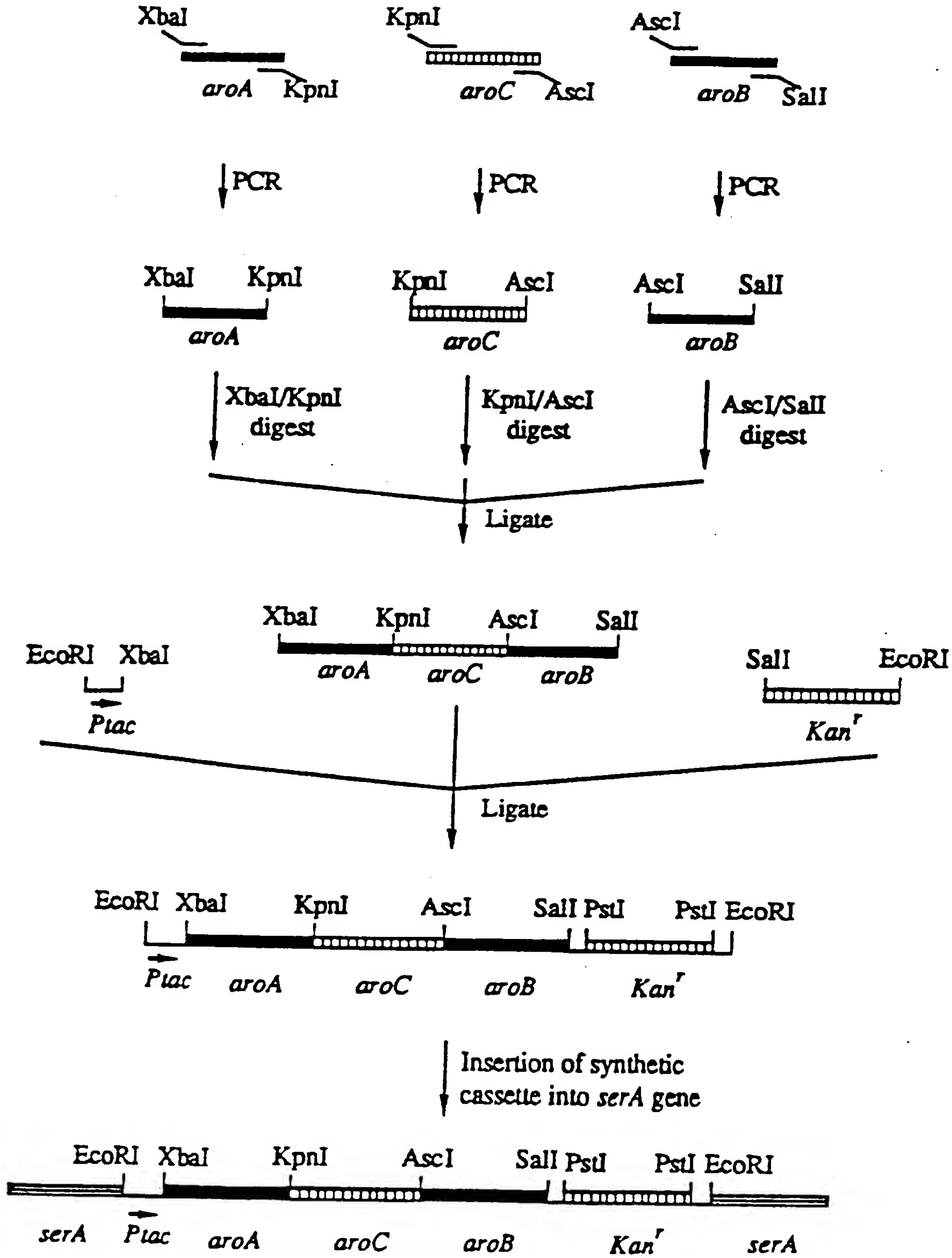

pKK223-3
4.585 kb
Ptac
amp
BamHI
HindIII PstI SalI BamHI SmaI EcoRI
pKAD38
4.7 kb
aroA
Ptac
cat
EcoRI
SacI
KpnI
PstI
SphI
HindIII
EcoRI+HindIII
EcoRI+HindIII
pKKaroA
6.9 kb
aroA
Ptac
KpnI
SacI
EcoRI
BamHI
HindIII SphI PstI
BamHI+HindIII
BamHI+HindIII
pAB17
7.7 kb
aroA
Ptac
aroL
aroB
ori
amp
Kpn
SacI
EcoRI
BamHI
RsaI
RsaI
HindIII SphI PstI
EcoRI
EcoRI

FIG. 15b



HindIII SphI PstI SalI
SmaI/ClaI
KpnI
SacI
EcoRI
Plac
aroC
pKAD39
4.0 kb
cat
PstI SalI BamHI SmaI EcoRI
HindIII
kan
pMB2190
4.5 kb
tet
amp
EcoRI SmaI BamHI SalI PstI
EcoRI
EcoRI
SmaI/ClaI
KpnI
SacI
EcoRI
SmaI
BamHI
SalI
PstI
HindIII
HindIII SphI PstI SalI
Plac
aroC
pKAD39kan
4.0 kb
kan
cat
EcoRI SmaI BamHI SalI PstI
HindIII
HindIII
BamHI
Plac
aroL
RsaI
HindIII
aroA
ori
pAB18B
9.4 kb
aroC
HindIII
aroB
amp
EcoRI
EcoRI

FIG. 15c



Design of Synthetic Cassette.

FIG. 16

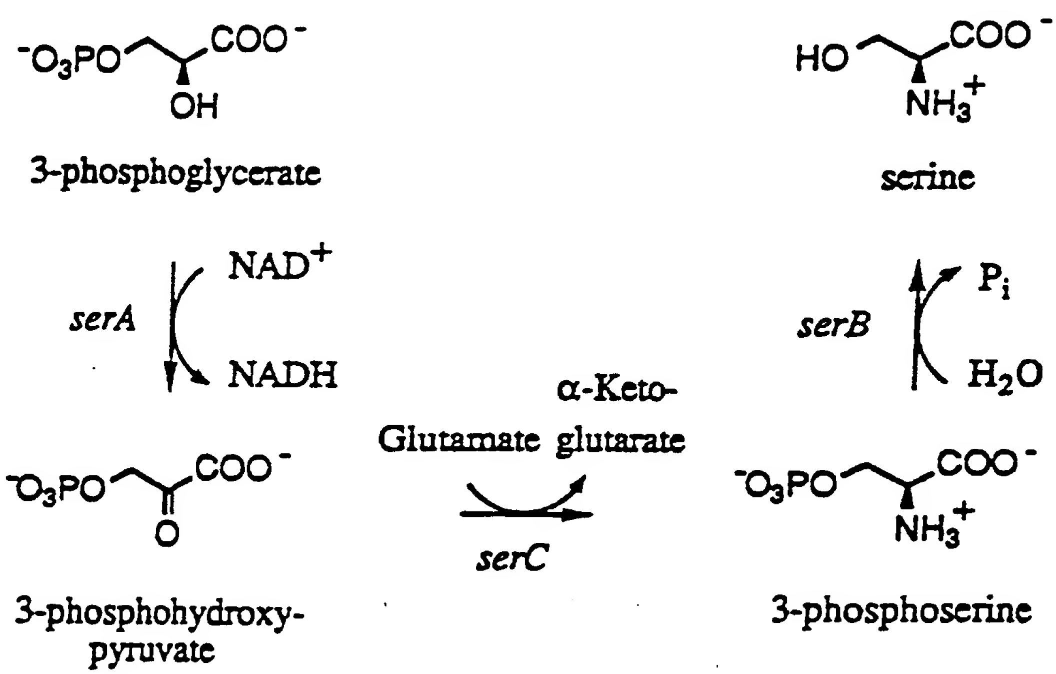

$^{-}O_3PO$
COO$^-$
OH
3-phosphoglycerate
serA
NAD$^+$
NADH
HO
COO$^-$
NH_3^+
serine
serB
P_i
H_2O
$^{-}O_3PO$
COO$^-$
O
3-phosphohydroxy-
pyruvate
Glutamate
α-Keto-
glutarate
serC
$^{-}O_3PO$
COO$^-$
NH_3^+
3-phosphoserine

FIG. 17

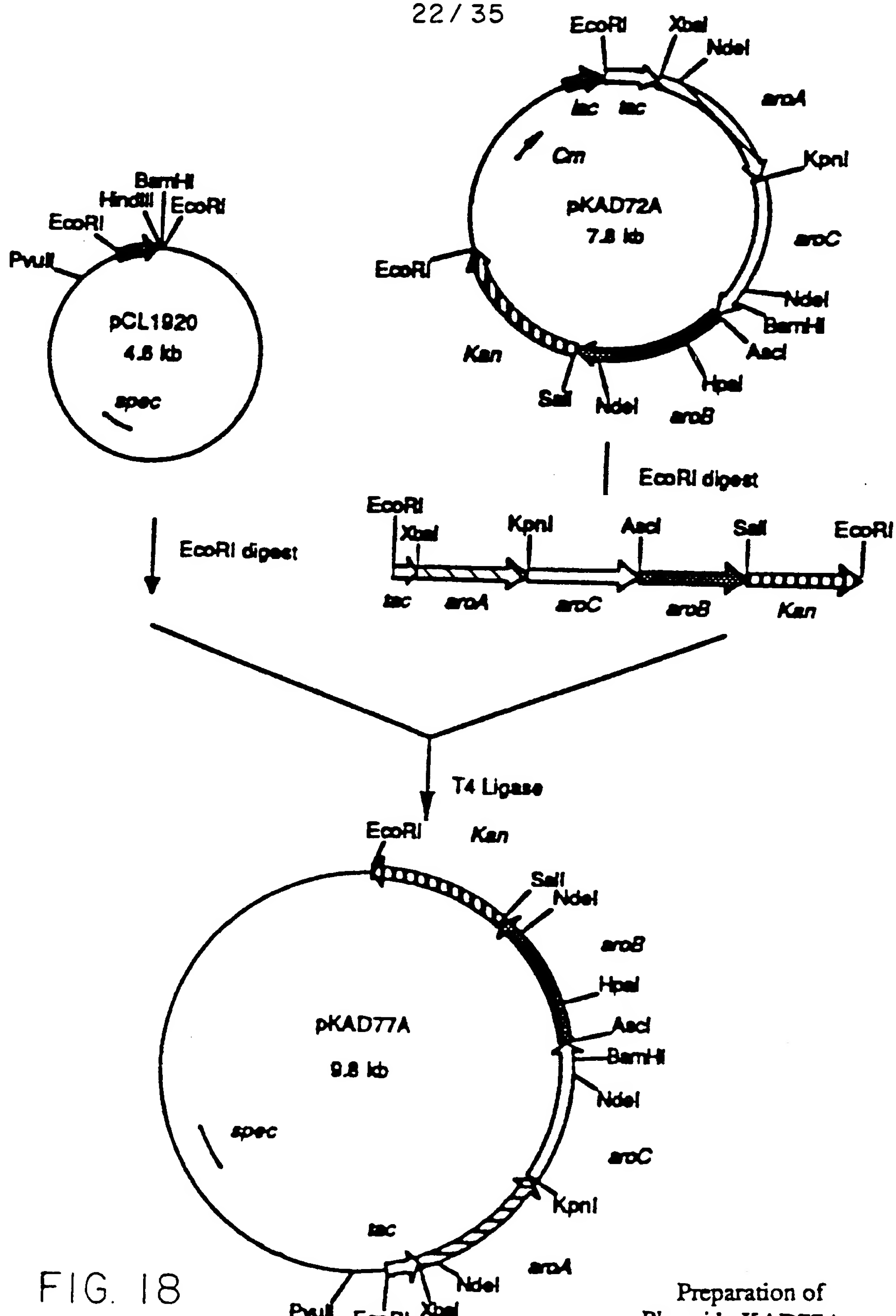


FIG. 18

Preparation of
Plasmid pKAD77A.

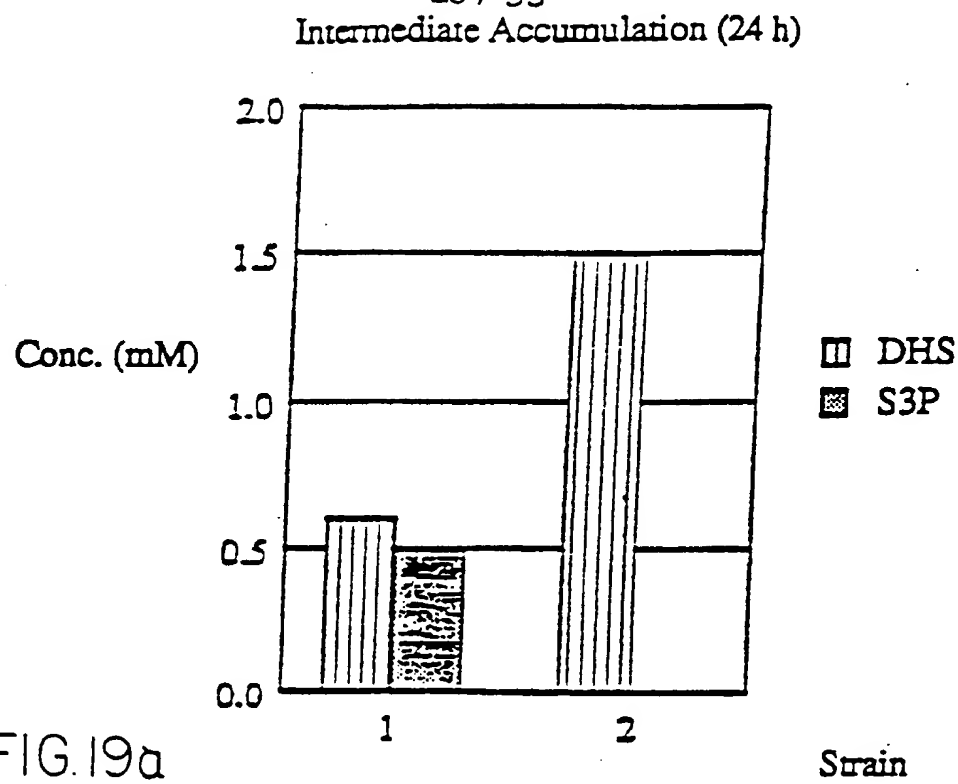


FIG.19a

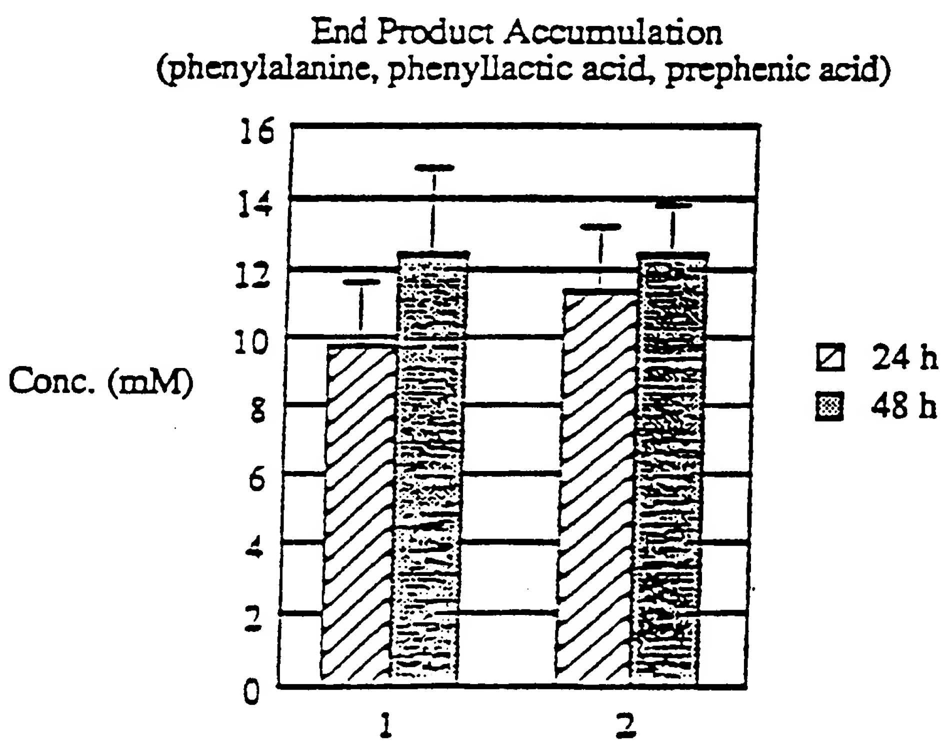


FIG. 19b

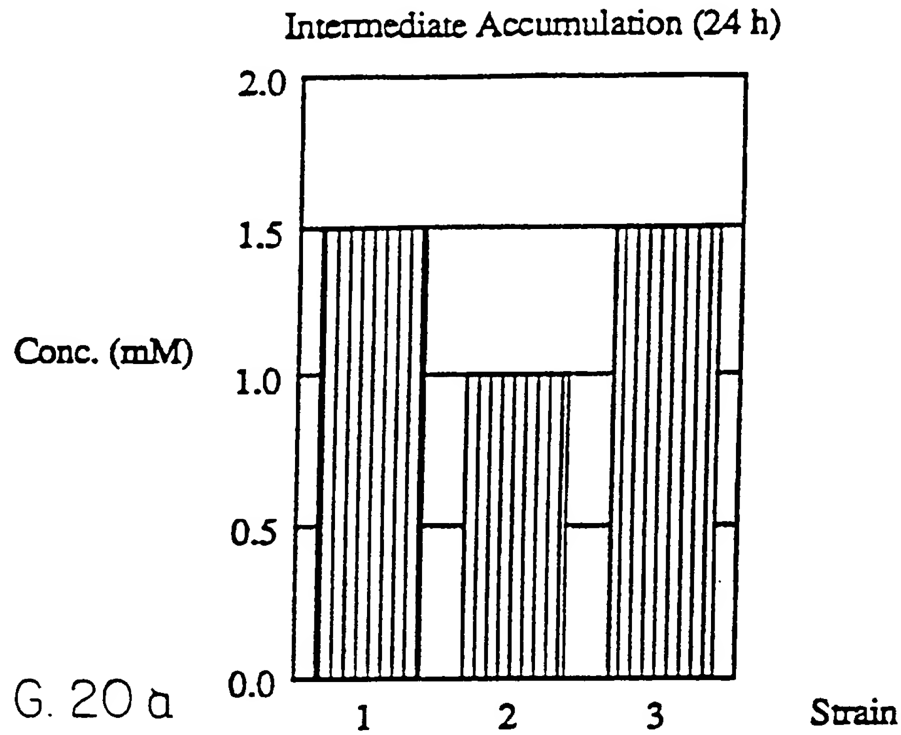


FIG. 20a

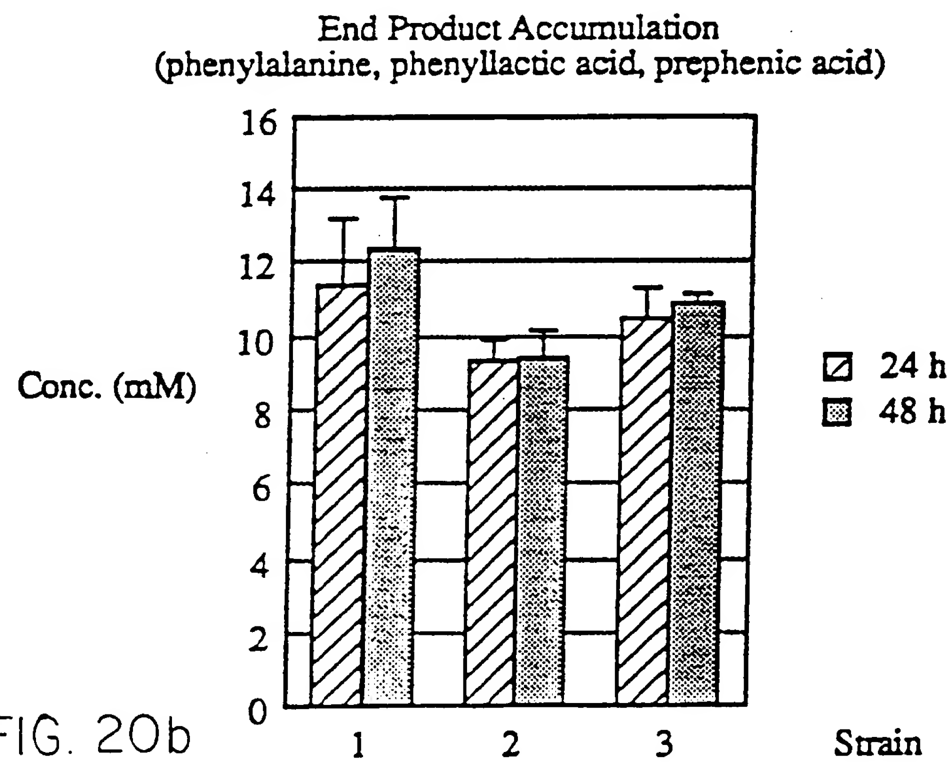
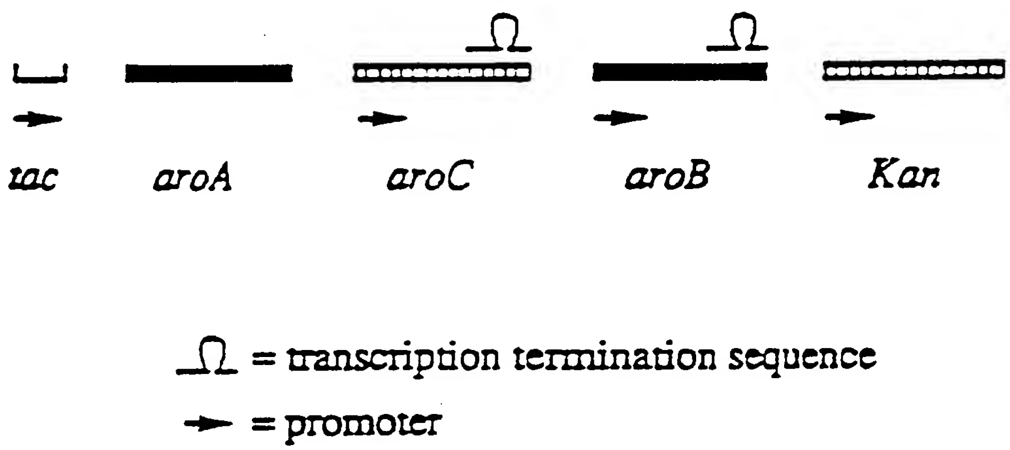


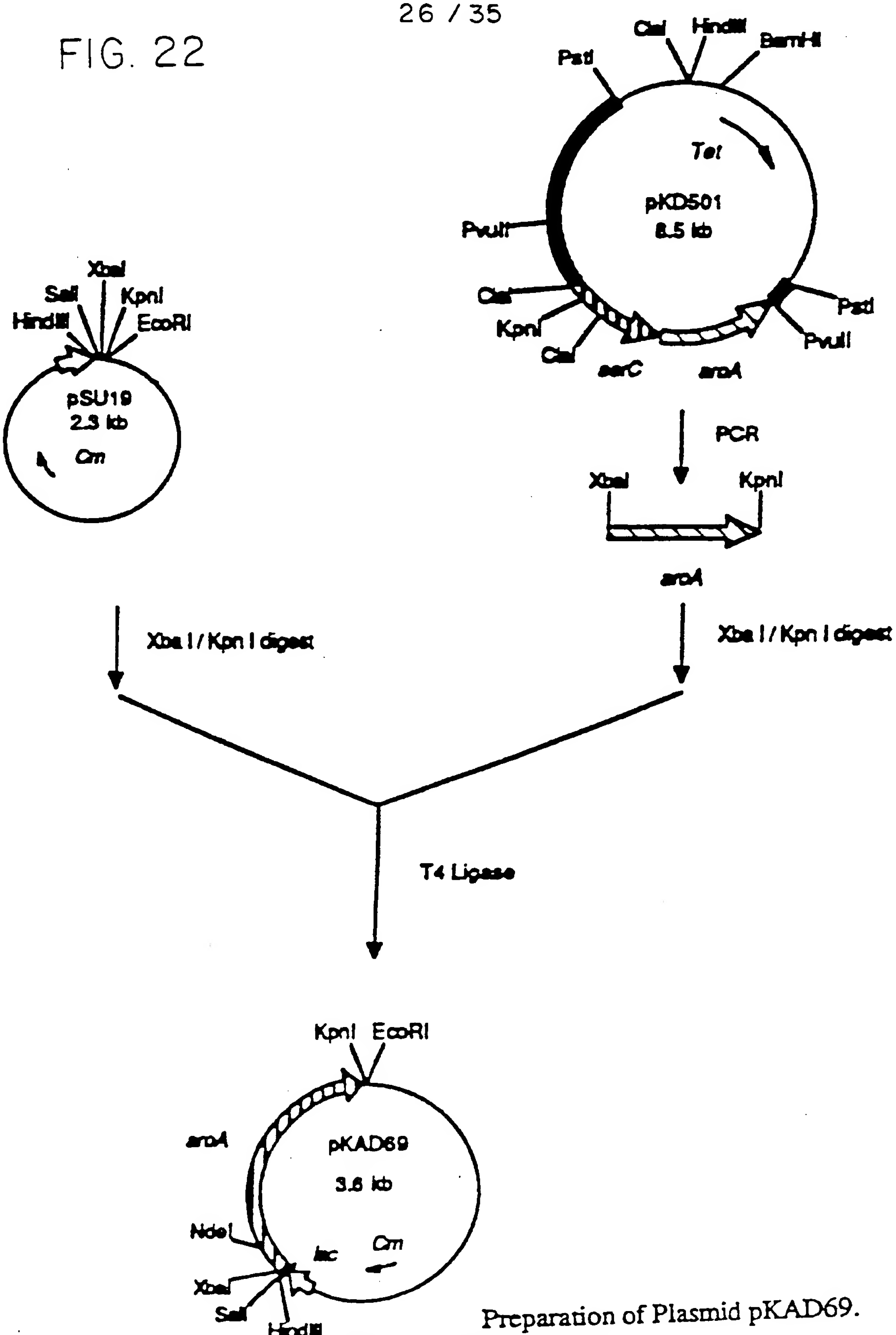
FIG. 20b



Location of Promoters and Transcription Termination Sequences in Cassette Fragments.

FIG. 21

FIG. 22



Preparation of Plasmid pKAD69.

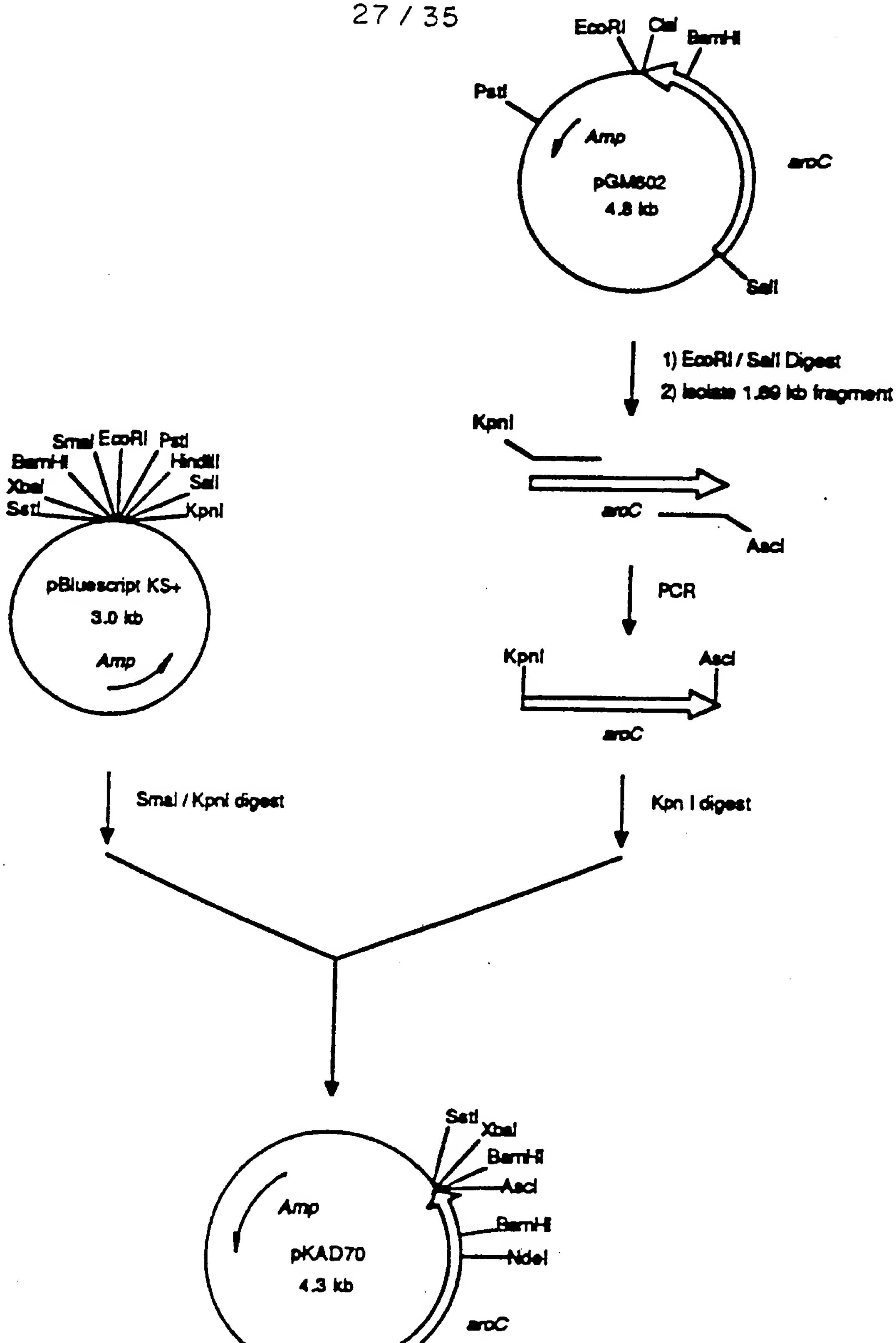
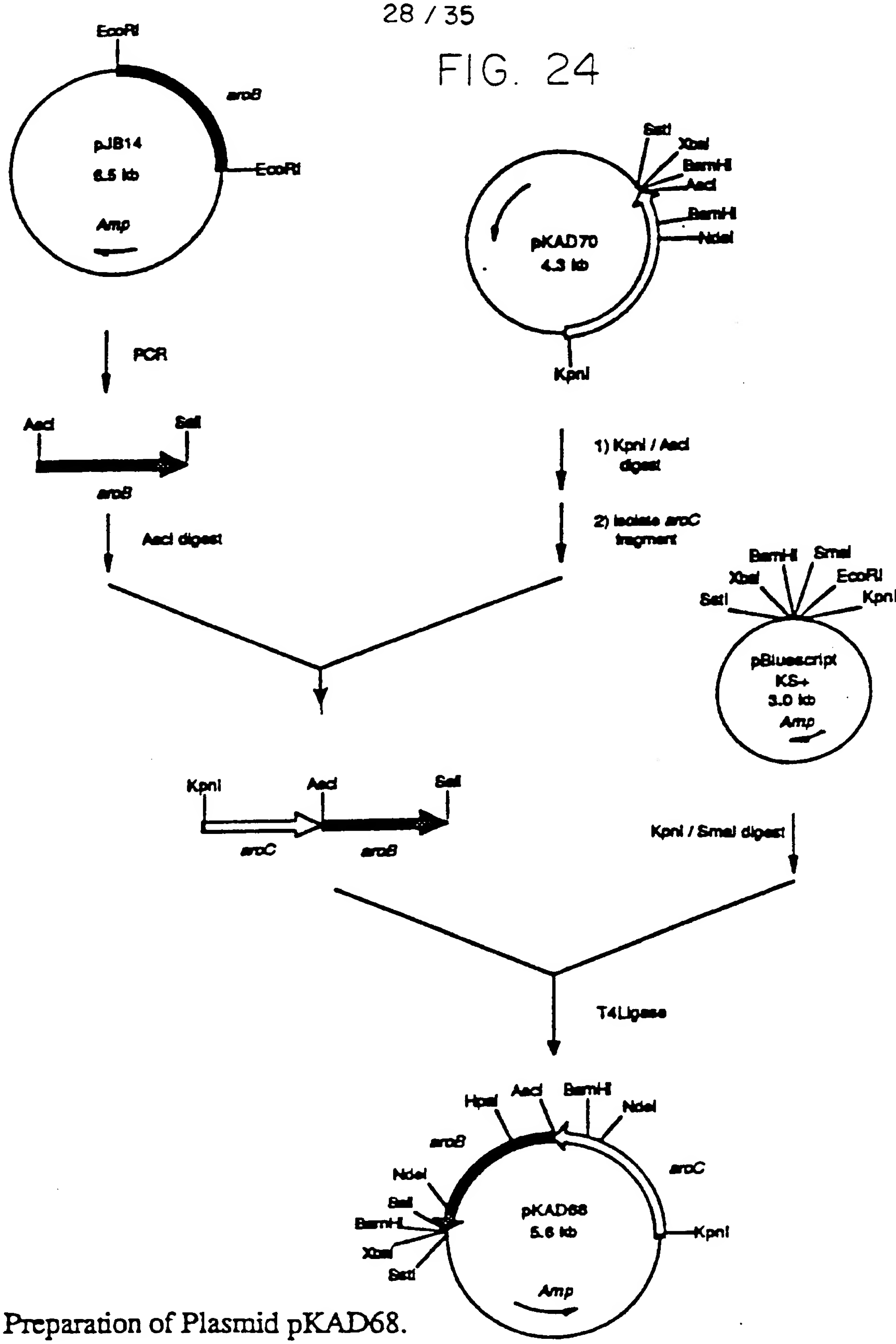


FIG. 23

Preparation of Plasmid pKAD70.

FIG. 24



Preparation of Plasmid pKAD68.

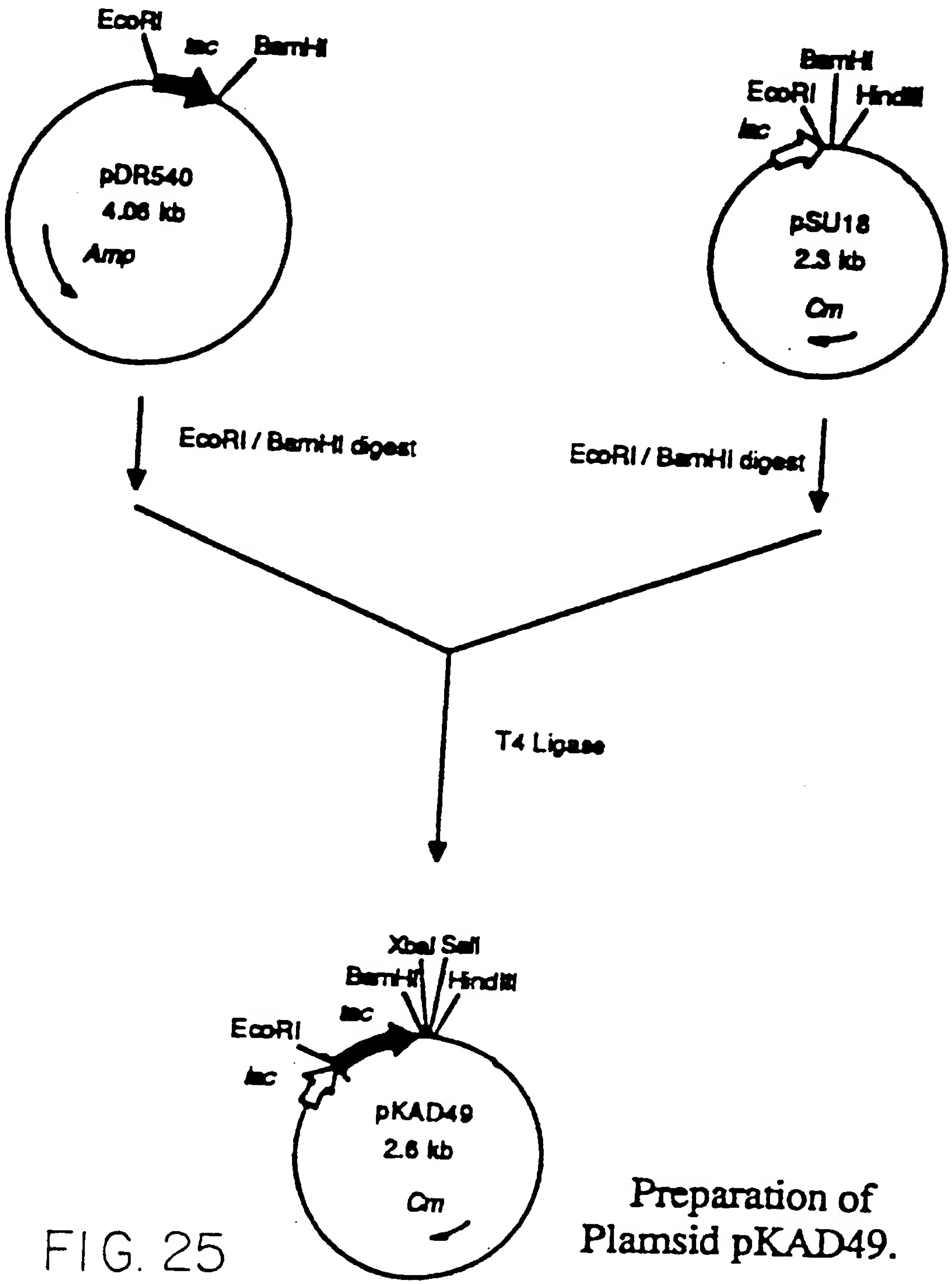


FIG. 25

Preparation of Plamsid pKAD49.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07321

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y ---- A	US, A, 5,304,475 (KIM ET AL.) 19 APRIL 1994, column 5, lines 15-38.	5 ---- 1-4, 6-9
Y ---- A	US, A, 4,681,852 (TRIBE) 07 JULY 1987, column 63, lines 1-25.	5 ---- 1-4, 6-9
Y ---- A	US, A, 4,980,285 (SANO ET AL.) 25 DECEMBER 1990, column 14, lines 56-68.	5 ---- 1-4, 6-9
Y ---- A	US, A, 5,017,481 (MATSUI ET AL.) 21 MAY 1991, column 9, lines 4-14.	5 ---- 1-4, 6-9

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 AUGUST 1995	29 AUG 1995

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Lisa J. Hobbs
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07321

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12P 13/22, 21/06, 21/04; C12N 15/31, 1/20, 9/10, 15/70

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/108, 172.3, 252.33, 183, 69.1, 71.2, 320.1

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

435/108, 172.3, 252.33, 183, 69.1, 71.2, 320.1

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN (Biosis, Biotechds, Ca, Cancerlit, Confsci, Dissabs, Embase, Jicst-E, Lifesci, Medline, Scisearch)
search terms: Dehydroquinate synthase, Shikimate kinase, Enolpyruvoylshikimate, Chorismate synthase, Transketolase, Arabino heptulosonate, Clone#, cDNA#, DNA#, Sequence#, Transform##, Transfect##

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07321

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P ---- A,P	US, A, 5,407,824 (KATSUMATA ET AL.) 18 APRIL 1995, column 14, lines 45-61.	5 ---- 1-4, 6-9
A	US, A, 5,168,056 (FROST ET AL.) 01 DECEMBER 1992.	1-4, 6-9